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OUR COVER

The cover, taken from the 2nd manuscript (Figure 1) published in this issue, is a Vernonia cineria (L.) Less., commonly called "tagulinai" among the Philippine medicinal plants.

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Anti-inflamatory Principles of the Leaves of Persea americana Mill.

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ABSTRACT

The ethanol extract of the dried leaves of Persea americana Mill exhibited anti-inflammatory activity when tested by the carrageenan-induced edema method, inhibiting the inflammation by about 76% at a dosage of 3 g/kg body weight. From the hexane and ethylacetate fractions of the extract was obtained after a bioactivity-directed fractionation scheme, an isolate HV_{6b} *which inhibited inflammation by about 66% at a dosage of 0.2 g/kg body weight and also reduced platelet aggregation induced by* $CaCl_2$ *Structure elucidation techniques employing infrared,* ^1H *and* ^{13}C *nuclear magnetic resonance and mass spectroscopy indicated that isolate* HV_{6b} *was avocadene [1] or 16-heptadecene-1,2,4-triol.*

From the ethylacetate fraction was also obtained another isolate $EIII_{2a}$ *which inhibited carrageenan-induced inflammation by 37% at same dosage. Preliminary characterization studies suggest that* $EIII_{2a}$ *is a flavonoid.*

INTRODUCTION

In Philippine folklore medicine, the leaves of *Persea americana* Mill., commonly known as avocado alligator pear, are used as a remedy for diarrhea, dysentery, abdominal pains, neuralgia, headache, rheumatism, gout, and high blood pressure (Ticson, 1986; Sas, 1984; Guttierez, 1980; Quisumbing, 1978). An infusion of the leaves is also said to have the following properties: anti-odontalgic, antimalarial, antihelmintic, diuretic, pectoral, rubefacient, and abortifacient. The flowers and leaves are taken as a substitute for tea. The seeds when roasted and ground, are used against retention of urine and as poultice over inflammations (de Padua et

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al, 1978; Cortes-Maramba, 1980; Concha, 1978). The oil obtained from the fruit is also a treatment for rheumatism, jointaches and neuralgia (Co and Taguba, 1989). Some local folks claim that a decoction of the leaves is a cure for arthritis.

Several compounds have been previously isolated from the seeds and fruits (Bittner et al, 1971; Brown, 1973) and one of the isolates, avocadene, has been shown to exhibit antibacterial activity. Another study confirmed the hypertensive action of the plant (Padmawinata, 1980).

On the basis of the traditional use of the leaves as treatment for rheumatism, arthritis, and jointaches , this study was undertaken to test the anti-inflammatory activity of the leaf extracts and identify the bioactive constituents which may provide, in part, scientific basis for its folklore use. Bioactivity-directed fractionation by rapid column chromatography yielded 2 active isolates.

This is the first report on the isolation of the anti-inflammatory-active constituents of the plant.

MATERIALS AND METHODS

Plant Material

Mature green leaves of *Persea americana* were collected at the campus of the University of the Philippines Diliman and air dried. A voucher specimen was deposited at the Herbarium of the Institute of Biology, University of the Philippines Diliman with herbarium no. 0001750-53.

Extraction and Column Chromatographic Fractionation

Dried leaves (1.157 k) were cut into small pieces and extracted with distilled ethanol. The ethanol extract was concentrated at 40°C under reduced pressure. The concentrated extract was partitioned between solvents of varying polarity (hexane, ethylacetate and butanol). The hexane and ethylacetate fractions were separately fractionated by rapid column chromatography using repeated gradient and isocratic reduced pressure elution following a bioactivity-directed scheme. Silica gel columns (Si Gel 60G, Merck) of varying sizes were used and eluted by gradient elution with hexane, then hexane-ethylacetate mixtures of increasing polarities, then ethylacetate, and finally with 2% methanol-ethylacetate. $\mathbf{HV}_{6b}$ was obtained from the active fraction **HV** after isocratic elution with 45% ethylacetate in hexane while $\mathbf{EIII}_{2a}$ was obtained from active fraction **EIII** after isocratic elution with 70% ethylacetate in hexane. Chromatographic separation was monitored by thin layer chromatography using pre-coated Si gel plates (Merck). The spots were visualized by spraying the plates with vanillin-concentrated sulfuric acid followed by heating in the oven for 5 minutes. The column fractions were recrystallized using acetone-hexane solvent system.

Bioassay for Anti-inflammatory Activity : Carrageenan-induced Edema Method (Winter et al, 1962; Buttle et al, 1957)

White mice, weighing 25 g to 35 g, were fasted for 16 hours prior to the experiment. A 2% carboxymethylcellulose (Sigma), which was used as solvent for indomethacin and the test extracts, was injected to the 1st group of mice as negative control. Indomethacin (Merck, Sharp and Dohme) was given to a 2nd group of mice as the positive control at a dosage of 0.01 g/kg body weight. The plant extracts were administered orally by a feeding gavage to a 3rd group of mice, at a dosage of 3 g crude extracts/ kg mouse or 0.2 g of TLC-pure isolates/kg mouse, one hour before intradermal injection of 0.04 ml 2% carrageenan (Sigma) in physiologic saline solution/25 g mouse. The volume of the paw was measured before carrageenan was injected and again, 3 hours after injection, using a fabricated plethysmometer. The % inhibition of inflammation was calculated as follows :

$$\% \text{ inhibition} = \frac{(\text{change in edema volume}_{CMC} - \text{change in edema volume}_{sample}) \times 100}{\text{change in edema volume}_{CMC}}$$

Statistical Analysis

The edema volumes were expressed as mean ± standard deviation (S.D.). The difference in paw volumes were analyzed statistically using Analysis of Variance and the Hypothesis Test for means.

Bioassay for Anti-inflammatory Activity: Platelet Aggregation (Born and Cross, 1963)

Platelet-rich plasma (PRP) was obtained from freshly extracted porcine blood treated with 6% dextran solution. Platelet aggregation was induced by a 1% $CaCl_2$ solution Aspirin, a known anti-inflammagen, was used as positive control. The PRP treated with $CaCl_2$ and with plant extracts were smeared on glass slides, stained successively with May-Grunwald and Giemsa solutions and observed under a high-power microscope. The extent of aggregation in each set was graded as follows:

(-) no aggregation
(+-) slight aggregation
(+) less aggregation than PRP plus $CaCl_2$
(++) as much aggregation as PRP plus $CaCl_2$
(+++) more aggregation than PRP plus $CaCl_2$

General Characterization and Structure Elucidation Procedures

The melting points ($\mathbf{HV_{6b}}$: 64.5°C; $\mathbf{EIII_{2a}}$: 263°C) were determined using a Mel-Temp II melting point apparatus. The UV spectrum of $\mathbf{HV_{6b}}$ (ν_{max} : 203 nm, 223 nm) was recorded with a Perkin-Elmer UV-Vis 551

Spectrophotometer while the UV spectrum of $\mathbf{EIII}_{2a}$ (ν_{max} : 350 nm) was taken with a Hitachi U-2000 UV-Vis Spectrophotometer. The IR spectra were recorded in KBr pellet using a Hitachi Infrared Spectrophotometer Model 270-30 ($\mathbf{HV}_{6b}$: 735, 845, 915, 1015, 1135, 1385, 1435, 1470, 1640, 2850, 2950, 3100, 3310 cm^{-}; $\mathbf{EIII}_{2a}$: 1045, 1080, 1385, 1485, 1620, 2895, 2950, 3450 cm^{-}). The NMR spectra of $\mathbf{HV}_{6b}$ were recorded with a Varian Unity 300 MHz Spectrometer in $CDCl_3$ (^1H NMR δ : 1.26, 1.58, 2.04, 3.48, 3.65, 3.89, 3.96, 4.99, 5.82; ^{13}C NMR δ : 25.41, 28.46, 28.74, 28.92, 29.14, 29.50, 29.63, 33.80, 38.12, 38.88, 66.69, 72.10, 72.66, 114.04, 139.21. A ^1H-^1H COSY, HMQC and an HMBC were likewise recorded. The MS spectrum (m/e: 287, 285, 269, 268, 255, 253, 237, 219, 211, 209, 135, 123, 121, 109, 105, 95, 87 (base peak, $C_4H_7O_2$), 83, 81, 69) was recorded with a V670-250S Double Focusing Magnetic Sector Mass Spectrometer

RESULTS AND DISCUSSION

Crude Extracts

The anti-inflammatory activities of the crude leaf extracts were established from the results of the carrageenan-induced edema method (Table 1). At a dosage of 3 g/kg body weight, the ethanol extract reduced inflammation by 75.6% while indomethacin at a dosage of 0.01 g/mg bodyweight by 70%.

Table 1. Inhibitory effects of extracts on Carrageenan-induced inflammation.

Test samples	Dosage g/kg body wt.	Edema volume x ± S.D.	% Inhibition
Carrageenan plus 2% CMC	–	0.082[1] ± 0.0125	–
Indomethacin	0.01	0.025[1] ± 0.0132	69.5
Ethanol extract	3	0.020[2] ± 0.0122	75.6
Hexane fraction	3	0.058[2] ± 0.0160	29.3
Ethyl acetate fraction	3	0.026[2] ± 0.0055	69.3
Butanol fraction	3	0.045[3] ± 0.0227	43.8

*[1]n=10; [2]n-6; [3]n=4

Of the 3 crude fractions obtained from solvent partitioning of the crude ethanol extract, the ethylacetate fraction was most effective in inhibiting inflammation, (68%) while hexane was the least effective with 29% inhibition. However, the hexane fraction was obtained in highest yield, 4.8%, as against 0.09% for the ethylacetate fraction and 0.14% for the butanol fraction (Fig. 1).

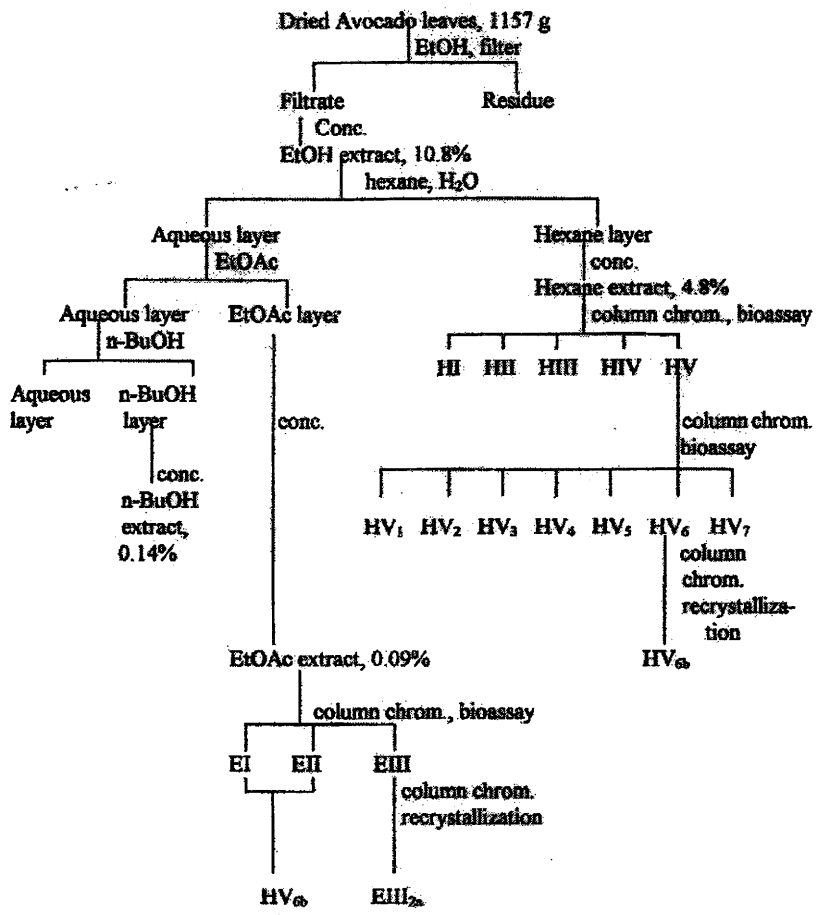


Figure 1. Extraction and fractionation scheme.

A bioactivity-directed scheme was employed to obtain isolates which exhibited anti-inflammatory activity. Rapid column chromatographic fractionation of the hexane fraction gave 5 fractions, HI - HV (Fig. 1). (Although low in activity, the hexane fraction was obtained in highest yield). Bioassay results indicated that fraction HV was active. Further chromatographic fractionation of active fraction HV gave seven fractions, HV_1 - HV_7. The major component of fraction HV, which appeared as white, needle-like crystals, was obtained in fraction $\mathbf{HV_6}$. Further purification by recrystallization in acetone-hexane gave TLC-pure $\mathbf{HV_{6b}}$.

On the other hand, rapid column chromatographic separation of the ethylacetate extract gave 3 fractions, EI - EIII. From the TLC results, fractions EI and EII were shown to contain similar compounds found in fraction HV of the hexane extract with the major component of similar

TLC characteristics as isolate HV_{6b}. From active fraction **EIII**, which reduced carrageenan-induced inflammation by 46% at a dosage of 3 mg/g body weight, was obtained TLC-pure isolate $EIII_{2a}$.

The analysis of variance was employed to compare the central tendencies or means of the inhibitory effects of the six groups of treated mice against each other. The following statistical values were obtained:

Calculated F-ratio		Tabulated F-ratio	
5%	1%	5%	1%
26.067	26.067	2.48	3.68

At both 5% and 1% confidence levels, the calculated F-ratios are greater than the tabulated ratios suggesting that the differences among the mean values of the six groups are highly significant. The probability value of 4.895×10^{-11}, which is much less than 0.01, further supports this inference.

The hypothesis test for means (Table 2) was also used to determine the statistical significance of the effect against edema response. The following values were obtained:

Table 2. Hypothesis test for means of crude extracts.

	$T_{calculated}$	$T_{tabulated}$	Probability
Control vs. indomethacin	9.8257	2.101	5.849×10^{-9}
Control vs. ethanol extract	9.6659	2.145	7.103×10^{-3}
Control vs. hexane extract	3.4179	2.145	2.081×10^{-3}
Contrl vs. ethyl acetate extract	10.2404	2.145	3.477×10^{-3}
Control vs. butanol extract	3.9821	2.179	9.097×10^{-4}

The calculated T values of indomethacin and the five extracts are greater than their respective tabulated T values at 0.05 significance level, confirming the significant differences between the mean value for the control and the means of the treatments. Their probabilities are much less than 0.01

Isolate HV_{6b}

Isolate HV_{6b} reduced inflammation by 66.3 % at a dosage of 0.2 g/kg body weight with no observed exudated liquid at the point of injection .

Table 3. Effects of isolate HV_{6b} on Carrageenan-induced inflammation.

	Dosage (g/kg body wt.)	Edema volume x ± S.D.	Inhibition (%)
Control (2% CMC)		0.095 (n=4) ± 0.0332	
Indomethacin	0.01	0.028 (n=4) ± 0.0050	70.53
Isolate HV_{6b}	0.2	0.032 (n=4) ± 0.0126	66.32

Results of the analysis of variance are shown in Table 4.

Table 4. Analysis of variance for pure isolates.

	Calculated F-ratio		Tabulated F-ratio	
	5%	1%	5%	1%
Control vs. HV_{6b}	13.227	13.227	4.26	8.02
Control vs. $EIII_{2a}$	9.255	9.255	4.26	8.02

At both 5% and 1% significance levels, the calculated F-ratios are greater than the tabulated F-ratios. Together with the values from the hypothesis test for means (Table 5) the inhibitory action of the isolate was confirmed :

Table 5. Hypothesis test for means for pure isolates.

	$T_{calculated}$	$T_{tabulated}$	Probability
Control vs. indomethacin	4.0249	2.447	3.460×10^{-3}
Control vs. HV_{6b}	3.5238	2.447	6.23×10^{-3}
Control vs. $EIII_{2a}$	3.000	2.447	1.200×10^{-2}

Isolate HV_{6b} was also observed to reduce the aggregation of platelets induced by $CaCl_2$ (Table 6) to the same extent as aspirin, a known anti-inflammagen.

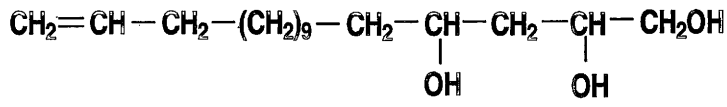
Table 6. Effect of HV_{6b} on platelet aggregation.

Test solution (2000 µg/mL)	Effect on platelet aggregation
PRP alone	−/+−
PRP + $CaCl_2$	++
PRP, $CaCl_2$ & aspirin	+−
PRP, $CaCl_2$ & V_{6b}	+−

Isolate $\mathbf{HV_{6b}}$ appeared as colorless, needle-like crystals with a melting point of 64.5°C. It is soluble in hexane, ether, chloroform, and carbon tetrachloride. On TLC, it appeared as a violet spot after spraying with vanillin-concentrated sulfuric acid and heating, with an R_f value of 0.26 in ethylacetate-hexane (7:3) developing solvent.

The MS showed a peak at m/e 287 which was taken as the molecular ion + H^+ while the peak at m/e 268 was the molecular ion of 286 with the loss of water. These suggested a molecular formula of $C_{17}H_{34}O_3$. The UVspectrum, on the other hand, showed absorption maxima at 223 nm and 203 nm, characteristic of acyclic compounds with double bonds while the IR spectrum suggested the presence of an aliphatic compound (2950 cm^- and 2850 cm^-) with a hydroxyl group (3310 cm^-) and a terminal double bond (1640 cm^- and 9115 cm^-).

The physical and MS and IR spectral data of $\mathbf{HV_{6b}}$ agreed closely with the data reported in the literature (Bittner et al. 1971; Brown 1973) for 16-heptadecene-1,2,4-triol **[1]**. Data from COSY, HMBC, and HQMC gave the carbon-proton connectivities which confirmed the structure of avocadene.



16-heptadecene-1,2,4-triol (avocadene) [1]

Isolate $\mathbf{EIII_{2a}}$

Isolate $EIII_{2a}$ reduced inflammation by 36.6% at a dosage of 0.2 g/kg body weight (Table 7). This inhibitory effect was found to be statistically significant as shown in the F & T values in Tables 4 and 5.

Table 7. Effects of isolate $EIII_{2a}$ on Carrageenan-induced inflammation.

	Dosage (g/kg body wt.)	Edema volume x ± S.D.	Inhibition (%)
Control (2% CMC)		0.071 (a) ± 0.0160	
Indomethacin	0.01	0.026 (a) ± 0.0189	63.38
Isolate $EIII_{2a}$	0.2	0.045 (a) ± 0.0071	36.62

Isolate $\mathbf{EIII_{2a}}$ appeared as a yellow solid with a melting point of 263°C. On TLC, it gave a yellow orange spot with vanillin-concentrated sulfuric acid/heat with an R_f = 0.50 with 10% methanol in ethylacetate solvent. The ethanolic solution of $\mathbf{EIII_{2a}}$ gave a reddish pink color upon reaction with hydrochloric acid and magnesium ribbon indicative of a positive

result in the Wilstatter "Cyanidin" test for the benzopyrone nucleus of flavonoids like those present in flavones, isoflavones, flavonols, and flavonones (Guevara et al, 1985). The UV spectrum gave a maximum absorption at 350 nm, typical of flavonoids, flavone and flavonol (Markham; Harborne, 1973), Visibly, flavones and flavonols are pale to bright yellow in color (Robinson, 1983) similar to isolate **$EIII_{2a}$**. On the other hand, the IR spectrum of **$EIII_{2a}$** showed stretching frequencies of the following bonds: O-H (3450 cm^{-1}) , C-H of methyls (2950 , 2895 cm^{-1}), C-O (1045, 1080 cm^{-1}) and C=O (1620 cm^{-1}) . The in-plane bending vibrations of the O-H bonds are at 1385 and 1485 cm^{-1}.

Isolate **$EIII_{2a}$** however gave a negative result for the Bate-Smith & Metcalf test which detects leucoanthocyanins. Leucoanthocyanins belong to a class of flavonoids which have leucoanthocyanidin as their aglycones.

These preliminary characterization results suggest that **$EIII_{2a}$** may be a flavonoid of the flavone or flavonol type. Further spectral analysis will confirm the identity of **$EIII_{2a}$**. The wide therapeutic applications of flavonoids include inhibition of inflammation (Havsteen, 1982).

The presence of both **HV_{6b}** and **$EIII_{2a}$** in the ethylacetate fraction could account for the higher activity of the ethylacetate than the hexane fraction, which contained only **HV_{6b}**.

CONCLUSION

The anti-inflammatory activity of the extract of the leaves of *Persea americana* was confirmed by the carrageenan-induced edema test. At a dosage of 3 g extract per kg body weight, the crude extract inhibited inflammation by about 76 %. No exudated liquid or other signs of irritation were observed in the injection area which indicated that the anti-phlogistic action of *P. americana* was not due to an irritating effect at the injection site.

The bioactivity-directed fractionation scheme led to the isolation of active compounds **HV_{6b}** and **$EIII_{2a}$** which reduced carrageenan-induced inflammation by 66% and 37 % respectively, at a dosage of 0.2 g/kg mouse. **HV_{6b}**, which was identified to be avocadene or 16-heptadecyne-1,2,4-triol **[1]**, also caused less platelet aggregation compared to the negative control and to about the same extent as aspirin. Preliminary studies suggested that $EIII_{2a}$ may be a flavonoid of the flavone or flavonol type.

Carrageenan, an algal polysaccharide, acts as an inflammagen by accelerating the generation of protaglandins which are important mediators contributing to the signs and symptoms of inflammation. The anti-inflammatory activity of non-steroidal drugs like aspirin and indomethacin has been attributed to their inhibitory effects on prostaglandin biosynthesis

by suppressing the formation of endoperoxides. Aspirin is also known to stop the production of thromboxane A_2 which form from endoperoxides and blocks the release of ADP stimulated by collagen thus, inhibiting the aggregation of platelets (Di Rosa, 1972; Bevan, 1976; Winter et al, 1966; Bowman et al, 1980; Zeller et al, 1983) and decreasing the release of serotonin and adenyl compounds.

The anti-inflammatory principle of *Persea americana* may, thus, be attributed to the combined effects of the two isolates, avocadene and the flavonoid, both of which seem to inhibit synthesis of prostaglandin.

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Diuretic Principles/Compounds from *Vernonia cinerea (L.) Less**

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ABSTRACT

The water soluble fraction (Fraction E) of the methanol extract of the defatted dried ground whole plant of Vernonia cinerea (L.) Less. showed significant diuretic activity in rats comparable to Lasix (Furosemide U.S.P.), a known diuretic. This fraction was found to contain terpene glycosides and potassium chloride. Comparison of the diuretic activity of Fraction E with the equivalent content of potassium chloride showed that Fraction E is more effective and this could be due to the synergistic effect of the glycoside and potassium chloride. Electrolyte excretion of the rats five hours after treatment with different doses of Fraction E indicated increase in potassium, sodium and chloride excretion respectively compared with the control.

INTRODUCTION

In the Philippines, more than 200 plant species are used as diuretics in folklore medicine (Quisumbing, 1951). Included among this is *Vernonia cinerea* (L.) Less., locally known as *tagulinai* or *tagulinau*, a common weed in open places throughout the archipelago at low and medium altitudes (Merrill, 1926). The plant is characterized by small flower heads in open lax corymbs; the flowers are bright purple, about 20 in each head (Figure 1) (Merill, 1912).

In Chuta Nagpur, India, the whole plant is used as a remedy for spasms of the bladder and for strangury or painful urination. In Senegal and French Guinea, an infusion of the plant is given to children with incontinence of urine (Quisumbing, 1951).

The following plant constituents of medicinal importance have been isolated from or identified as present in *Vernonia cinerea:* luteolin 7-0 glucoside, luteolin 7-0 galactoglucoside and quercetin 7-0 glucoside 3-0 rhamnoside, all flavonoids (Wagner, H. et.al., 1972 and Mabry, T.J., et.

*Paper read at the Malaysian Silver Jubilee Chemical Congress, Federal Hotel, Kuala Lumpur, November 18, 1992.

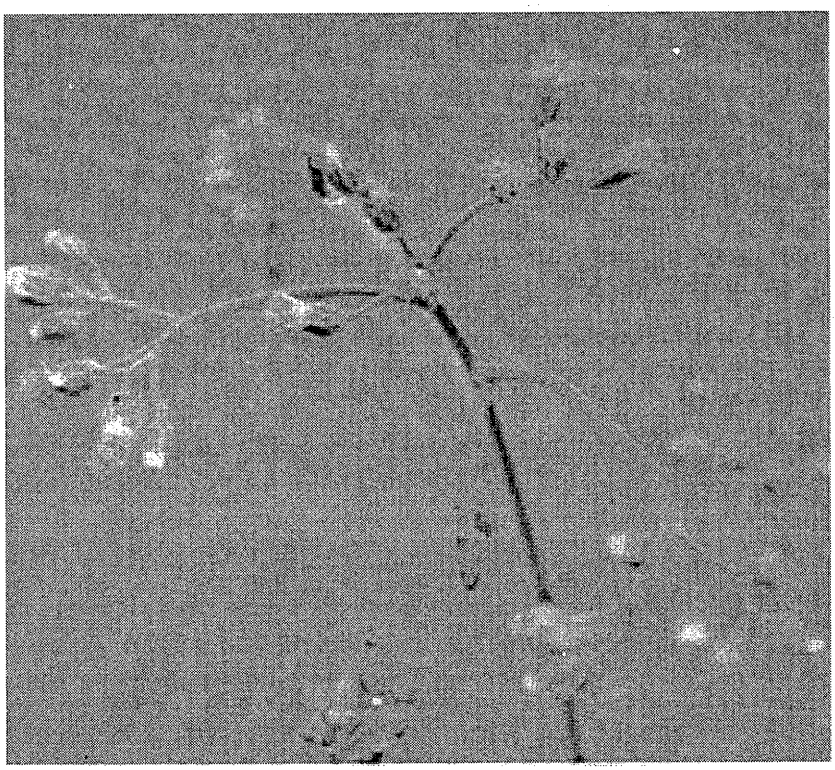

Figure 1. *Vernonia cinerea* (L.) *Less.*, commonly called "tagulinai."
(Reprinted from: de Padua, L.S., G.C. Lugod and J.V. Pancho. 1981. Handbook on Philippine Medicinal Plants. Vol 3. U.P. Los Baños, p. 17)

al., 1975) pentaynene, an unsaturated long chain hydrocarbon (Bohlmann,F. et.al., 1972); and epoxy oleic acid or vernolic acid (Earl,F.R., 1970). Flavonoids were reported to possess diuretic properties (Williams, 1964).

MATERIALS AND METHODS

Extraction. The air-dried, powdered whole plant of *V. cinerea* collected from Metro Manila, Philippines were extracted successively with petroleum ether (b.p. 30°- 60°C) and anhydrous methanol in a Soxhlet extractor.

Fractionation of Methanol extract is shown in Figure 2. The methanol extract was extracted with hot water until the last extract was colorless. The aqueous extracts were filtered. The water insoluble fraction was dried and coded as Fraction A. The filtrate was extracted with chloroform until the last extract was colorless. The dried chloroform fraction was coded as Fraction B. The aqueous layer was extracted with ethyl acetate and the dried ethyl acetate fraction was coded as Fraction C. The aqueous layer was exhaustively extracted with butanol and the dried butanol fraction was

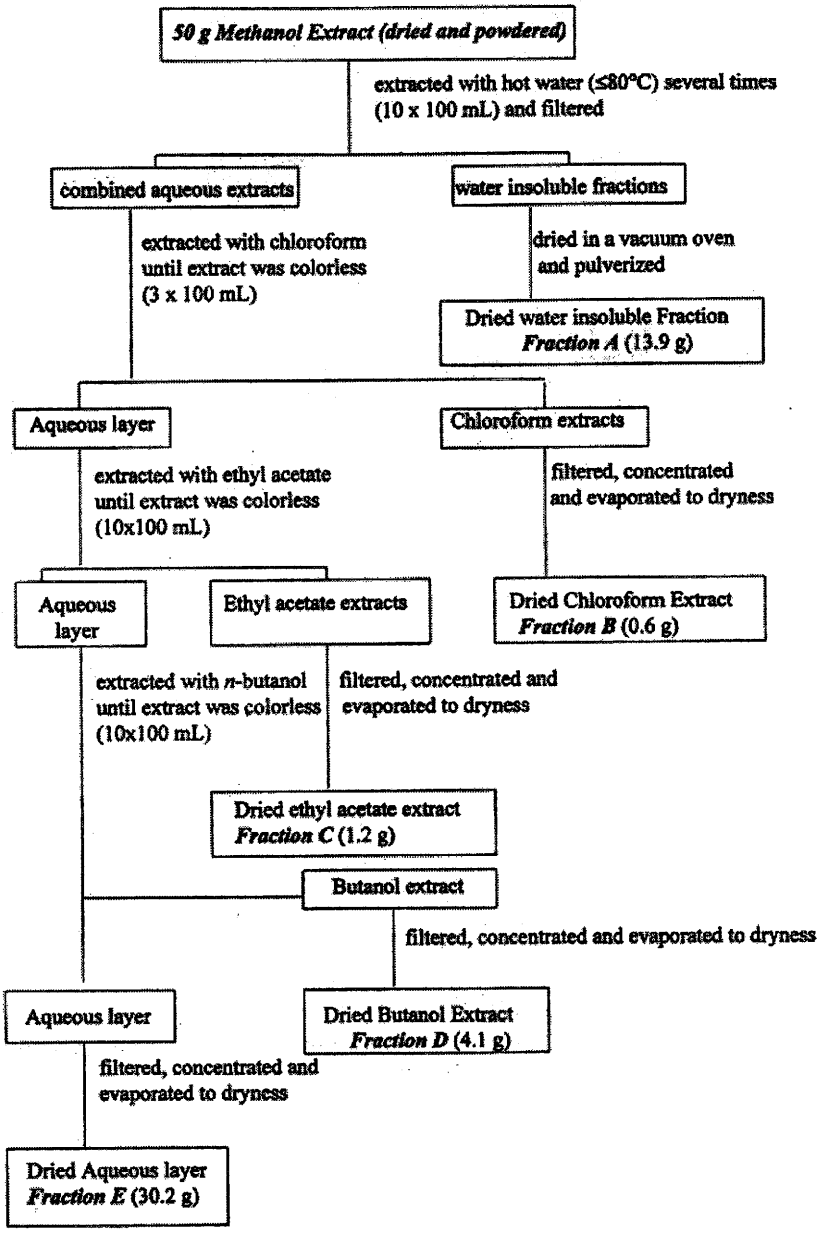
50 g Methanol Extract (dried and powdered)
extracted with hot water (≤80°C) several times (10 x 100 mL) and filtered
combined aqueous extracts
water insoluble fractions
extracted with chloroform until extract was colorless (3 x 100 mL)
dried in a vacuum oven and pulverized
Dried water insoluble Fraction Fraction A (13.9 g)
Aqueous layer
Chloroform extracts
extracted with ethyl acetate until extract was colorless (10x100 mL)
filtered, concentrated and evaporated to dryness
Aqueous layer
Ethyl acetate extracts
Dried Chloroform Extract Fraction B (0.6 g)
extracted with n-butanol until extract was colorless (10x100 mL)
filtered, concentrated and evaporated to dryness
Dried ethyl acetate extract Fraction C (1.2 g)
Butanol extract
filtered, concentrated and evaporated to dryness
Aqueous layer
Dried Butanol Extract Fraction D (4.1 g)
filtered, concentrated and evaporated to dryness
Dried Aqueous layer Fraction E (30.2 g)

Figure 2. Fractionation of the methanol extract of "*tagulinai*."

coded as Fraction D. The residual aqueous layer was evaporated to dryness over a water bath and further dried in a vacuum oven. This fraction was coded as Fraction E.

In later fractionations, chloroform was omitted as extracting solvent since the yield was not enough for diuretic activity testing.

Purification of the Water Soluble Fraction or Fraction E is shown in Figure 3. The water soluble fraction, Fraction E was refractionated to separate impure KCl as follows:

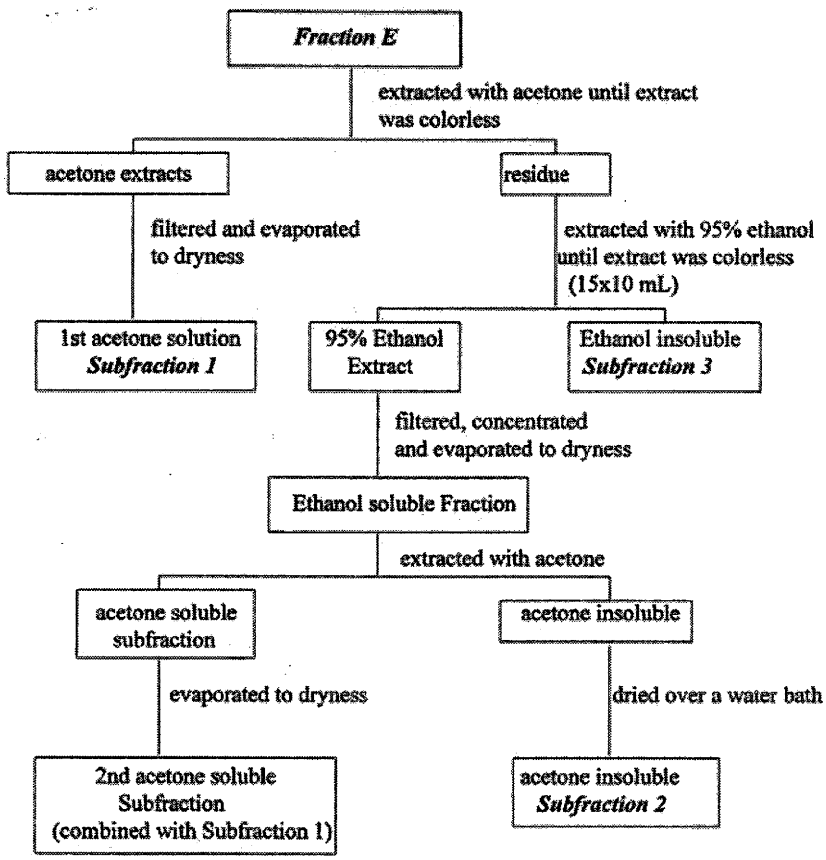

Figure 3. Purification of fraction E of the methanol extract of "*tagulinai*."

Fraction E was pulverized and exhaustively extracted with acetone. The acetone extracts were filtered and evaporated to dryness, coded as Subfraction 1. The residue was extracted with 95% ethanol until the extract was colorless. The ethanol insoluble fraction was dried and coded as Subfraction 3. The ethanol extracts were filtered and evaporated to dryness. This ethanol soluble fraction was extracted with acetone until colorless. This second acetone fraction was combined with the first acetone fraction obtained. The acetone insoluble, ethanol soluble fraction was dried and coded as Subfraction 2.

Diuretic Activity Testing Method

Male and female Sprague-Dawley rats weighing 180-300 g each were used as test animals. These were fasted and deprived of water for 18 hours before the experiment and randomly grouped in separate metabolic cages provided with urine-feces separators (3 rats/group). The extracts/fractions of *V. cinerea* suspended in 10% Tween 80 in normal saline solution (NSS) were administered orally, after which NSS alone was given (saline loading). The negative control groups received a similar volume of the saline loading vehicle without the extract as well as the positive control groups with 7 mg/kg body weight Lasix (4-chloro-N-2furyl-methyl-5 sulphamylanthranilic acid/furosemide U.S.P.), a potent diuretic (Gennaro et.al., 1985). The dose of Lasix used was the predetermined minimum effective dose in rats. The urine of the test animals were collected in graduated centrifuge tubes every hour for 5 hours.

RESULTS AND DISCUSSION

Results of diuretic activity testing of the methanol extract is shown in Table 1. Results indicate that the crude methanol extract at a dose of 1,000 mg/kg body weight is almost as effective as Lasix at a dose of 7 mg/kg. LD_{50} of the extract was found to be 2,224 mg/kg body weight in mice (Herrera, et.al., 1978).

Table 1. Diuretic activity of the methanol extract of "tagulinai" in rats (Average of 3 trials).

Treatment	Urinary excretion mL/kg/3 hours
Negative control	2.4 ± 0.49
300 mg/kg	4.8 ± 0.33
700 mg/kg	5.5 ± 0.14
1000 mg/kg	9.5 ± 1.15
Lasix at 7 mg/kg	8.9 ± 2.25

Qualitative analysis of the methanol extract showed the presence of terpenes, tannins, flavonoids, glycosides, sugars and pigments (Herrera et.al., 1978).

Fractionation of the methanol extract yielded the components present in the different fractions as shown in Table 2. The butanol soluble and the residual water soluble fractions contain most of the components as shown in the table. The residual water soluble fraction or Fraction E contain glycosides and potassium chloride but no flavonoids while the butanol fraction contains all these components. Results of the diuretic activity of the different fractions shown in Table 3, indicate that the residual water

Table 2. Qualitative test of the different fractions of the methanol extract of "tagulinai."

Fraction	Glycoside	Chloride	Terpenes	Tannins	Flavonoids
water insoluble	–	–	+	–	–
chloroform soluble	–	–	+	–	–
ethyl acetate soluble	–	–	+	–	–
butanol soluble	++	+	++	+	+
residual water sol.	++	+++	++	–	–

Legend: +traces; ++moderate amounts; +++abundant

Table 3. Diuretic activity testing of the different fractions of "tagulinai" methanol extract in rats.

Subfraction	Volume of urine after 5 hours (mL)				
	250 mg/kg	500 mg/kg	1000 mg/kg	Negative Control	Positive Control*
A-water insoluble	1.2	2.1	0.8	2.9	6.0
B-ethyl acetate soluble	3.6	0.7	3.5	1.0	7.6
D-butanol soluble	1.5	4.6	6.0	1.8	3.6
E-water soluble	1.5	4.6	7.6	1.2	4.6

*Lasix at 20 mg/kg

soluble fraction is the most active followed by the butanol soluble fraction. Several trials done on the diuretic activity testing of Fraction E, shown in Table 4 indicate significant diuretic activity. Since the aqueous fraction was found to contain terpenes, glycosides and chlorides, this was further purified by refractionation as shown in Figure 3. Qualitative test of the

Table 4. Diuretic activity of different doses of Fraction E of "tagulinai" in rats (Average of 3 trials).

Drug/Dose	Volume of urin 5 hours ± S.E.
Normal saline solution	1.033 ± 0.57*
Lasix at 20 mg/kg	10.47 ± 5.47*
Fraction E at 250 mg/kg	2.20 ± 0.90*
Fraction E at 500 mg/kg	6.60 ± 1.48*
Fraction E at 1000 mg/kg	14.47 ± 4.35*

*Significant at 5% and 1% levels

subfraction shown in Table 5, indicate that most of the potassium chloride content of Fraction E can be separated by treatment with acetone followed by ethanol. The ethanol insoluble fraction consists of crude KCl which was purified by decolorization with activated charcoal and recrystallization. Results of diuretic activity testing shown in Table 6, indicate that the most active is Subfraction 3, or the ethanol insoluble subfraction followed by Fraction 2, the acetone insoluble subfraction. The physico-chemical properties of the isolated compound are shown in Table 7.

Table 5. Qualitative test of the subfractions of Fraction E extract of "tagulinai" methanol extract.

Subfraction	Glycoside	Terpenes	K^+	Cl^-
acetone soluble	–	+	–	–
ethanol soluble, acetone insoluble	++	+	++	++
ethanol insoluble	–	–	+++	+++

Table 6. Diuretic activity testing of the subfractions of Fraction E of "tagulinai" methanol extract in rats (Average of 2 trials).

Subfraction	Volume of urine after 5 hours (mL)				
	250 mg/kg	500 mg/kg	1000 mg/kg	Negative Control	Positive Control*
Acetone soluble	4.3	3.4	5.2	0.6	5.4
Ethanol soluble, acetone insoluble	4.7	4.0	10.8	0.2	9.6
Ethanol insoluble	1.9	1.4	7.7	0	3.0

*Lasix at 20 mg/kg

Table 7. Physico-chemical properties of the isoloated compound from Fraction E of "tagulinai" methanol extract.

Properties	Isolated compound	Indications
Appearance	crystalline	similar to KCl A.R.
Color	light creamish white	
Reaction with $AgNO_3$ T.S.	white curdy precipitate insoluble in HNO_3 but soluble in slight excess of 6N NH_4OH	presence of chloride ion
Reaction with MnO_2 + H_2SO_4	evolution of Cl_2 indicated by blue color of starch iodine paper	presence of chloride ion
Color with non-luminous flame	purplish yellow	presence of potassium ion
Reaction with sodium bitartrate T.S.	white crystaline precipitate upon addition of ethanol and with stirring soluble in 6N NH_4OH and insoluble in 1N NaOH solution	presence of potassium ion

The salt excretion of the rats treated with different doses of Fraction E were determined. Results shown in Table 8, indicate that with increasing doses of Fraction E, there is a corresponding increase in the excretion of potassium, sodium and chloride.

Table 8. Electrolyte excretion of rats five hours after treatment with different doses of Fraction E.

Source of urine	Potassium	Sodium	Chloride	Volume of urine
Negative control	2.7 mg	1.14 mg	3.0 mg	1.5 mL
Lasix treated at 20 mg/kg	16.8 mg	21.7 mg	49.7 mg	7.0 mL
Fraction E treated at				
0.25 g/kg	20.5 mg	11.16 mg	24.2 mg	3.5 mL
0.50 g/kg	91.8 mg	42.30 mg	108 mg	9.0 mL
1.00 g/kg	109.9 mg	75.00 mg	164 mg	15.2 mL

The yields of the active fractions are shown in Table 9. The yield of Fraction E is 0.63% of the fresh whole plant or 3.5% of the ground dried material. The yield of potassium chloride is 0.3% of the fresh whole plant or 1.8% of the ground dried material.

Table 9. Yield of "tagulinai" active fractions.

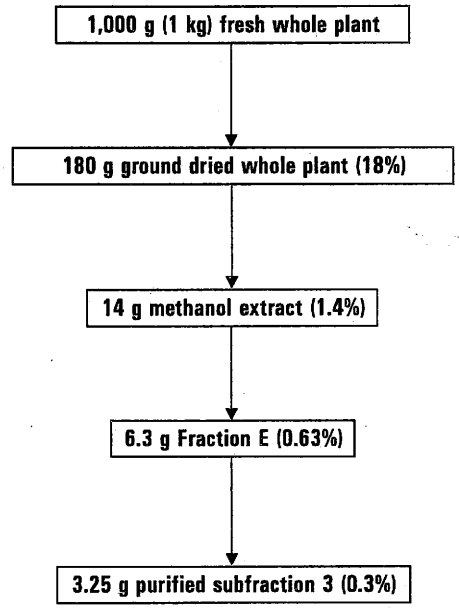
1,000 g (1 kg) fresh whole plant
180 g ground dried whole plant (18%)
14 g methanol extract (1.4%)
6.3 g Fraction E (0.63%)
3.25 g purified subfraction 3 (0.3%)

Table 10. Comparison of diuretic activity of Fraction E with an equivalent amount of potassium chloride.

Drug	Dose	Volume of urine after 5 hours	
		for July Batch	for May Batch
10% Tween 80 in NSS	–	0.7 mL	2.5 mL
Lasix	20 mg/kg	17.3 mL	28.3 mL
Tagulinai Fraction E	1000 mg/kg	15.0 mL	27.8 mL
Potassium chloride	500 mg/kg	11.0 mL	–
	400 mg/kg	–	16.8 mL

Since the amounts of the subfractions of Fraction E were quite small and not enough for more diuretic activity testing, Fraction E was assayed for KCl content. Two batches were assayed giving 51% and 40% KCl respectively. Diuretic activity tests were done on these comparing their activity with Lasix and commercial KCl in the same amount as assayed. Results as shown in Table 9 indicate that Fraction E is more active than a solution containing an equivalent amount of KCl. Based on the foregoing, we conclude that the other component of Fraction E which is the glycoside contributes to the activity of this fraction.

CONCLUSION AND RECOMMENDATIONS

The water soluble fraction of the methanol extract of the defatted dried whole plant of *Vernonia cinerea (L.) Less.* showed significant diuretic activity in rats comparable to Lasix at 7 mg-20 mg/kg body weight. This fraction contains KCl and a glycoside. Activity is due to the synergistic activity of the two components. Identification and/or structure elucidation of the glycoside component is recommended for further studies.

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Karyomorphometrics and Essential Oil Analysis in *Anisochilus* Wall. Ex Benth.

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ABSTRACT

Chromosome analysis conducted on Anisochilus carnosus (2n = 34) and A. eriocephalus (2n = 32) show specific micromorphometrical features. The presence of methyl iso eugenol, methyl eugenol, β-caryophyllene and β-selinene in both taxa reveal their phylogenetic relationship.

INTRODUCTION

Anisochilus Wall. Ex Benth. (Lamiaceae) is a cosmopolitan genus and holds about twenty species probably originated in India-Thailand and distributed mainly in the Indian subcontinent and tropical Africa (Fyson, 1986). Medicinal species like *A. carnosus* (L.f.) Wall. and *A. eriocephalus* Benth. are used as an expectorant, diaphoretic and to cure liver diseases (Anon, 1986).

Previous cytological studies on *Anisochilus* species are based on chromosome counts alone (Krishnappa and Basavaraj, 1982; Saggoo and Bir 1983). Detailed efforts has hitherto not been made to reveal the chromosome configuration and chemical constitution of these plants, in order to exploit their medicinal and aromatic potential. So in the present investigation, karyomorphometrical and gas liquid chromatography analyses have been conducted on *A. carnosus* and *A. eriocephalus*.

MATERIALS AND METHODS

Germplasm collections were made from the Western Ghats of India and grown under similar agro-climatic conditions. Herbarium specimens were prepared from voucher specimens at the Sacred Heart's College.

Karyomorphometrical analysis

Mitotic experiments were conducted on young healthy root tips pretreated with a mixture of saturated aqueous para-dichlorobenzene with a trace of aesculine and saponin at 0-5°C for 5 min. and then at 10 - 14°C for 2½ to 3 hrs. They were then fixed in Carnoy's fluid for 24 hrs., followed by the aceto-orcein squash techniques (Sharma and Sharma, 1980). Leitz Biomed photomicroscope and Kodak high contrast copy film served for

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microphotography. The centromeric index or Forma % (F%) of all the chromosomes were calculated as has been done by Krikorian *et al.* (1983)

$$(F\% = \frac{\text{Short-arm length}}{\text{Total chromosome length}} \times 100),$$

followed by mean centromeric index value or Total forma % (TF%). The Disparity Index (DI) of the chromosome complement was calculated according to Mohanty *et al.* (1991).

$$(D.I = \frac{\text{Longest chromosome - Shortest chromosome}}{\text{Longest chromosome + Shortest chromosome}} \times 100)$$

Essential oil characterization

The essential oil is isolated on a dry weight basis by hydro-distillation in a Clevanger apparatus for 4-5 hrs. at 100°C. GLC work was performed on a Chemito 8510 gas chromatograph equipped with flame ionization detector and connected to a Shimadzu (model-Chromatopac C-R3A) computing integrator. GLC conditions-column:stainless steel, 2.5 m x 3.0 mm (Internal Diameter), 80/100 mesh packed with 5% SE-30; operating conditions:temperature programmed from 80°C to 230°C at 6°c/min. and isothermal at 230°C, integrator and detector temperature 200°C; carrier gas: N_2, inlet pressure 10 psi, flow rate 30 ml/min.; sample injection: split ratio C. 1:100, sample volume 0.1 µl. The percentage composition of oil samples were computed from the GLC peak areas without using correction factors. Major components were analyzed by relative retention time analysis and peak enrichment by co-injection with authentic standards.

RESULTS AND DISCUSSION

The chromosome number was found to be 2n = 34 in *A. carnosus* (Fig. 1) and 2n = 32 in *A. eriocephalus* (Fig.2). The karyotype in both species of *Anisochilus* are characterized by the small size of the

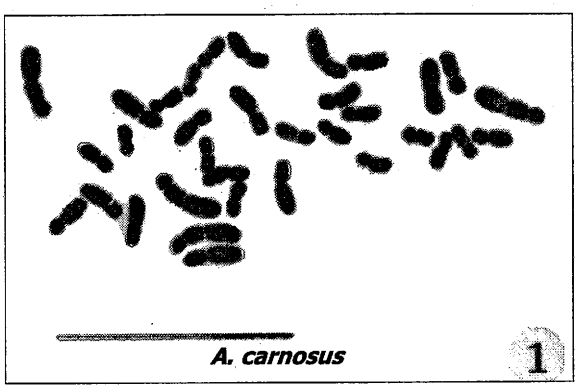
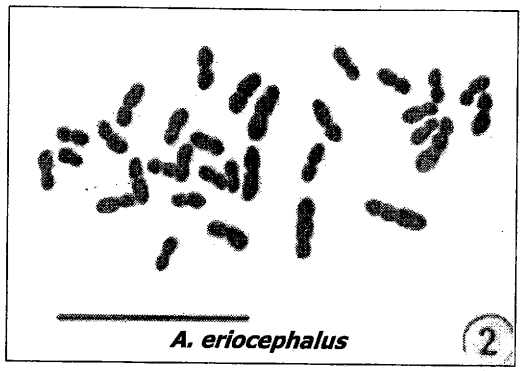


Figure 1&2. Chromosomes of two species of Anisochilus. (Bar=10µm)



chromosomes ranging from 3.4 to 1.2 mm in length. The average chromosome length was found to be 2.16 µm in *A. carnosus* and 1.91 µm in *A. eriocephalus*. Both the species, reveal the presence of Satellite-chromosomes (Type A: 3.4 - 2.8 µm), comparatively long chromosomes (Type B: 2.8 - 2.0 µm) and short chromosomes (Type C: < 2.0 µm). The karyotype formula was found to be A4B14C16 in *A. carnosus* and A4B12C16 in *A. eriocephalus*. The number of submetacentric chromosomes was 6 in *A. carnosus* and 10 in *A. eriocephalus*. In addition to this, they also exhibit remarkable karyomorphometrical differences. TCL, TF% and DI values were found to be greater in *A. carnosus*, when compared with *A. eriocephalus* (Table 1, Fig. 3).

Table 1. Summary of karyomorphometrical and chemical analyses in two species of *Anisochilus*.

Taxa	TCL (µm)	TF%	DI	Density of oil (20°)	Refractive index (20°)	Major essential oil components
A. carnosus	73.6	42.24	47.83	0.937	1.491	Methyl isoeugenol (19.4%), Carveol (13.3%) β-Caryophyllene (7.0%), β-Ionone (6.5%), Methyl eugenol (6.2%), β-Selinene (4.8%)
A. eriocephalus	61.2	41.45	45.45	0.941	1.4887	Methyl isoeugenol (16.9%), Dihydrocarvone (11.1%), β-Selinene (9.5%), α-Humulene (8.1%), β-Caryophyllene (5.4%), Methyl eugenol (5.3%)

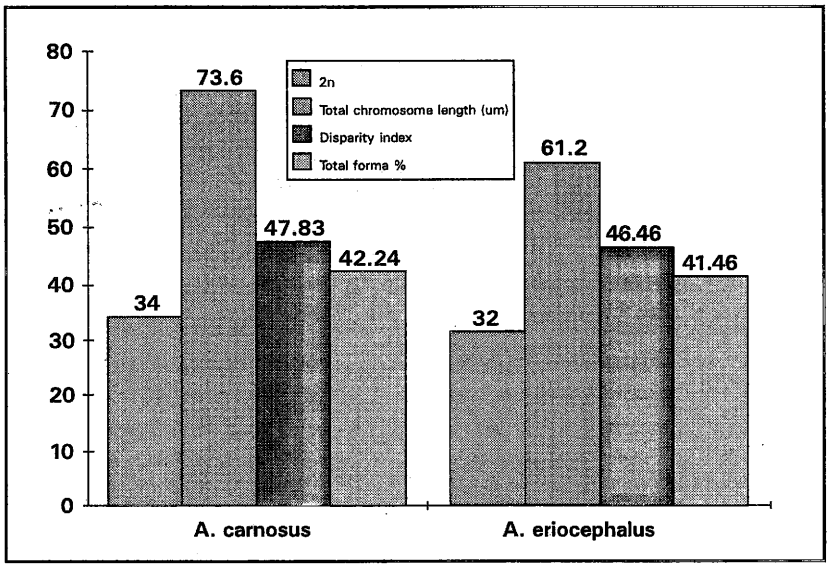


Figure 3. Comparisons of karyomorphometrical data in two species of Anisochilus.

Chromosome studies also reveal the existence of two different secondary basic chromosome numbers, one with X2 = 16 in *A. eriocephalus* (2n = 32) and the other with X2 = 17 in *A. carnosus* (2n = 34). The secondary base number of X2 = 16 might have arisen from the primary base number X1 = 8, through autopolyploidy (Fernandes and Leitao, 1984). Whereas, X2 = 17 might have originated from the primary base numbers X1 = 8 and X1 = 9 through amphipolyploidy (Sharma and Sharma, 1984) or from X1 = 9 through a 'polyploid drop' or from X1 = 8 through a 'polyploid lift' (Evans, 1988).

The comparatively higher karyomorphometrical values found in *A. carnosus* is of much significance with respect to it's evolutionary status. The high TF% value found in *A. carnosus* show the presence of a symmetrical karyotype. A comparatively high mean centromeric index value corresponds to the primitive status in flowering plants (Vasil'eva *et al.*, 1985). A decrease in total chromosome length (TCL) and average chromosome length along with the abundance of submetacentric chromosomes found in *A. eriocephalus* appears to be advanced karyomorphometrical features. Reduction in the chromosome length along with the subsequent increase in submetacentric chromosomes in the karyotype are highly advanced evolutionary characteristics (Sharma and Sharma, 1984).

The percentage yield of essential oil was found to be very low in both species of *Anisochilus* (0.1%). The brownish yellow coloured essential oil bears a spicy odour with a woody topnote. They possess a mildly warm flavour with a medicinal aftertaste. The essential oil was found to contain

monoterpenoids, sesquiterpenoids and phenolic derivatives (Table 1). In *A. carnosus*, phenols (methyl iso eugenol and methyl eugenol) were found to be the most significant group (25.6%), followed by the monoterpenoid group (19.8%) of compounds (carveol and β-ionone). Substantial amount (11.8%) of sesquiterpenes (β-caryophyllene and β-selinene) were also present (Table 1). Whereas, *A. eriocephalus* show the presence of phenols (methyl iso eugenol and methyl eugenol) which amounts to 23.2% of the total composition and sequiterpenes (β-selinene, α-humulene, β-caryophyllene) which form 23.0% of the total oil content. A comparatively lesser amount (11.1%) of monoterpenoids (dihydro carvone) sums up the major essential oil components in *A. eriocephalus* (Table 1). The chemical composition in both taxa seem to be remarkable. The presence of three different classes of chemical compounds reveal the presence of a mixed chemotype in both species of *Anisochilus*. The presence of similar classes of essential oil constituents denotes their phylogenetic relationship. Moreover, methyl iso eugenol, methyl eugenol, β-caryophyllene and β-selinene were found to be common in both taxa. The diploid symmetrical karyotype found in both taxa point towards their almost similar genetic constitution. This together with the chemical uniformity observed in the two species of *Anisochilus* is of much chemotaxonomic significance. Genetic control of essential oil constituents has been proposed on other members of Lamiaceae, viz. *Ocimum* (Thoppil and Jose, 1994) *Salvia* (Putievsky *et al.*, 1990) and *Coleus* (Thoppil and Jose, 1995). Moreover, it is well known that chemical characteristics in essential oil yielding plants are attributed to their genetic constitution (Lawrence, 1992).

In addition to these, the two species of *Anisochilus* also exhibit differences in both qualitative and quantitative aspects of other major constituents (Table 1). Chemical differences in closely related plants are attributed to their respective bioynthetic pathways established by heredity (Tetenyi, 1973).

SUMMARY

Anisochilus carnosus (2n = 34) and *A. eriocephalus* (2n = 32) possess a diploid, symmetrical karyotype with remarkable micromorphometrical differences. Both taxa belong to mixed chemotypes, with phenols, monoterpenoids and sesquiterpenoids forming the major classes of chemical compounds. Methyl isoeugenol, methyl eugenol, β-caryophyllene and β-selinene were found to be some of the common essential oil constituents. The similar classes of essential oil constituents denotes their phylogenetic relationship.

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Seedling Identification of Major Weeds Plantations of Fast Growing Tree Species in Mt. Makiling, Philippines I. Monocotyledons[1]

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ABSTRACT

The morphological characters of the seedlings of 11 species under 10 genera and 3 families of major monocotyledonous weeds in plantations of fast growing tree species in Mt. Makiling, Luzon, Philippines were studied. Observations on the external characters were used to describe and distinguish each species for easy recognition in the field. The species exhibited the hypogeal type of germination distinct from the epigeal type exhibited by the dicotyledonous weeds. Characters considered diagnostic for species identification include the mesocotyl, cotyledonary stalk, culm/ or stem and leaf. A seedling key to species identification is presented. Likewise, seedling description and illustration for each species are provided.

INTRODUCTION

Weed seedling identification is an important aspect in weed science aimed at helping crop managers control weeds and improve crop harvest. Controlling weeds at their seedling stage admittedly saves time, effort and resources. Further, mature weeds would certainly produce seeds that may be the source of future infestation and eventually offer competition detrimental to the favored crops (Dyatmiko and Robles, 1987).

Certain types of weeds are controlled most effectively using specific methods (Dalisay and Mercado, 1988; Cabangbang and Riyanto, 1989; Janiya et al, 1989). At present, the problem of weed control is addressed by selective chemical herbicides, cultural operation or practices, biological control and botanical herbicides or a mixture of these methods (Torres, 1990; Cruz 1990; Elliot and Moody, 1987; Janiya and Moody; 1987; Robles, 1991). Zindall et al (1990) have come up with a manual on the seeds and seedlings of weeds of rice in South and Southeast Asia. However, identification of weed seedlings in forest plantations and

[1]A portion of the author's MS Thesis submitted to the Graduate School, University of the Philippines at Los Baños, College, Laguna, Philippines

reforestation projects is likewise needed at present because weed control is admittedly one of the major factors that can spell out its success or failure.

This study was conducted to describe morphologically the seedlings of the major weeds found in plantations of fast-growing tree species in Mt. Makiling, Luzon, Philippines. The present report deals with the monocotyledonous weeds.

MATERIALS AND METHODS

Mature seeds of monocotyledonous weeds found in plantations of yemane (*Gmelina arborea* R. Br.), ipil-ipil [*Leucaena leucocephala* (Lam.) de Wit], [*Anthocephallus chinensis* (Lam.)Rich. ex Walp.], and Moluccan sau [*Paraserianthes falcataria* (L.) Nielsen] were collected and sown in seedboxes. They were allowed to germinate for observation. Herbarium materials of the mother plants were prepared following standard botanical procedure. These voucher materials are lodged at the Forest Products Research and Development Institute Herbarium (CLP)[2] and UPLB Museum of Natural History Herbarium (CAHUP)[3] for future reference.

The following species were studied:

1. *Cyperus kyllingia* Endl.
2. *Cyperus michelianus* (L.) Link.
3. *Dictyospermum vitiense* (Seen) J.K. Morton
4. *Aclisia secundiflora* (Bl.) Bakh.
5. *Eleusine indica* (L.) Gaertn.
6. *Centhoteca latifolia* (L.) Trin.
7. *Setaria palmifolia* (Koenig) Stapf
8. *Cyrtococcum oxyphyllum* (Steud.) Stapf
9. *Digitaria ciliaris* (Retz.) Koel.
10. *Rottboellia cochinchinensis* (Lour.) Clayton
11. *Paspalum conjugatum* Berg.

At least ten seedlings for each species were studied for the first and second stages of developmental growth. The first stage of growth referred to a seedling when the first leaf and first node and internode is visible for observation. The second stage referred to a seedling when the second and older leaves as well as the second and succeeding internodes are visible for observation. The seedlings were illustrated based on live specimens and the vegetative parts were described from root to tip. The terminology used in the seedling descriptions and key to identification were adopted from previous authors (Carrier, 1971; Hall, 1985; Hanf, 1970; Pancho et al, 1961,1963).

[2]International abbreviation of the FPRDI Herbarium, College, Laguna Philippines.
[3]International abbreviation of the UPLB Museum of Natural History Herbarium, College, Laguna, Philippines.

RESULTS AND DISCUSSION

The major diagnostic character distinguishing the monocot from the dicot weeds is the manner by which the cotyledons emerge from the seed. In the dicot, the cotyledons are emergent and borne by the erect hypocotyl above the soil level and are withdrawn from the seed by spreading (Escobin, 1988). The monocot weeds exhibited a non-emergent cotyledon, remaining in the soil inside the testa after germination.

Among species examined in this report, characters found diagnostic are as follows:

Mesocotyl

This segment is associated in the families Graminae and Cyperaceae. It is a segment inserted between the cotyledon sucker and the coleoptile (Fig. 1b). The mesocotyl is absent in *Dictyospermum vitiense* and *Aclisia secundiflora* while strongly developed and purplish in *Rottboellia cochinchinensis*; weakly developed in *Eleusine indica.*

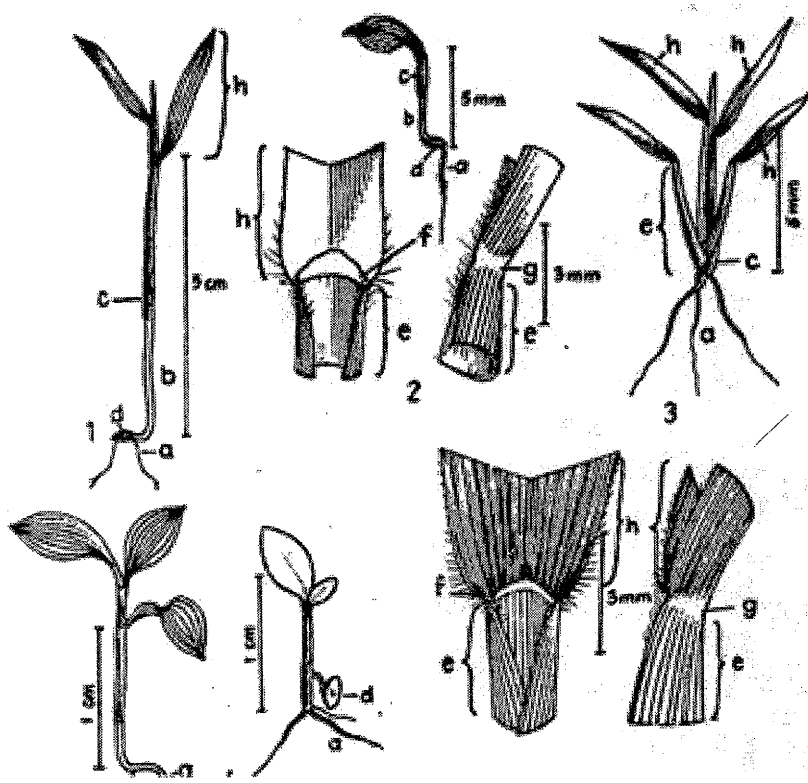

Figure 1. Nomenclature of seedling parts (monocot). a) root system; b) mesocotyl; c) coleoptile; d) cotyledon enclosed in seed; e) leaf sheath; f) ligule; g) collar; h) blade. *(1. Rottboellia cochinchinensis; 2. Cyrtococcum oxyphyllum; 3. Paspalum conjugatum; 4. Aclisia secundiflora)*

Cotyledonary Stalk

This structure is observed strongly developed in *Dictyospermum vitiense* and *Aclisia secundiflora* (Fig. 4 and 5). According to Arber (1961) this is a distinctive feature for the family Commelinaceae. In the cyperaceous species however, it is the coleoptile-mesocotyl axis which elongates on one side and the coleorhiza on the other side (Fig.1 and 2).

Leaf

The most useful clue for identification are provided by the leaf. The vernation is rolled in all the grasses examined but distinctly folded in *Eleusine indica.* The leaf sheath of the cyperaceous species are membranous and closed. In contrast, it is open in the grasses and commelinaceous species. The shape of the leaf sheath of the grasses in cross section supports earlier reports that it serves as a constant character (Pancho et al, 1961; Hall, 1985). In the grasses, it is either oval or circular in cross section but may be flat or keeled in *Eleusine indica* and *Paspalum conjugatum. Centhoteca latifolia* and *Eleusine indica* have glabrous leaf sheath while other grasses have hairs confined at the base as in *Cyrtococcum oxyphyllum* or scattered throughout the blade as in *Rottboellia cochinchinensis.* Generally the color is green to light green but in *Cyrtococcum oxyphyllum* and *Setaria palmifolia* it is distinctly pinkish to purplish.

The absence or presence of hairs in the leaf collar is also diagnostic. It is glabrous in *Centhoteca latifolia* while with hispid or soft hairs in the other grasses. The ligule also offers feature distinctive for the species. The membrane may be free of hair as in most of the grasses examined or fringed with hairs at the margin as shown in *Setaria palmifolia.* The membrane is entire in *Centhoteca latifolia* and toothed or jagged in *Digitaria ciliaris* and *Eleusine indica.*

The relative pubescence of the upper and lower surfaces of the leaf blade is also considered diagnostic. The seedlings of *Dictyospermum vitiense* and *Aclisia secundiflora* are so similar that they can be distinguished only in minor details of the leaf blade. The first leaf of the former is scabrous on both surfaces and distinctly larger than the latter having its first leaf scabrous only above. In the grasses, the blades are distinctly glabrous in *Centhoteca latifolia* and scabrous in *Rottboellia cochinchinensis.*

Culm/or stem

The cyperaceous species are distinguished by their triangular culm as seen in cross section while species under Graminae and Commelinaceae have circular culm/or stem.

Seedling key to species[4]

The following seedling key to identification of species is based on the above cited diagnostic characters selected from the descriptions prepared. It is highly artificial and presented in bracketed style. Each couplet is composed of two contrasting headings (or leads) under the same left-hand number. The first couplet to be consulted stands at the head of the key next to the number 1. The next appropriate couplet to consult is indicated by a reference number to further down the key, placed on the right-hand side of the chosen lead.

1 (para)cotyledons[5], 2 emergent, borne by the erect hypocotyl above the soil surface and are withdrawn from the seed by spreading .. **dicotyledonous weeds (not included in this report)**

(Para)cotyledon absent, cotyledons non-emergent, remaining in the soil inside the testa 2

2 Culms triangular in cross section 3

Culms circular in cross section 4

3 First leaf 2.5 cm x 1 mm, blade glabrous, margin scabrous; roots white to creamy white ..... 1. *Cyperus kyllingia* Endl. (mutha)

First leaf 5 cm x 0.4 mm, all parts glabrous; root brownish .....

..... 2. *Cyperus michelianus* (L.) Link (bala-balangutan)

4 Leaves without ligules 5

Leaves ligulate 6

5 Leaf blades scabrous on both surfaces leaf sheaths light green to green with tinge of purple; bearded at the outer margin; first leaf 1.5 x 1.0 cm

..... 3. *Dictyospermum vitiense* (Seen) J.K. Morton (alikbangon)

Leaf blades scabrous above; leaf sheaths light green to green, not bearded at the outer margin; first leaf 0.6 x 0.4 cm .....

..... 4. *Aclisia secundiflora* (Bl.) Bakh. (lohod-lohod)

6 Leaves folded in the bud, edges of leaf buds do not overlap..... 5. *Eleusine indica* (L.) Gaertn. (paragis)

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[4]Names in parenthesis refer to the popular local names of the species. Numbers opposite species names indicate their order of appearance in the subsequent descriptions.

[5]This term was proposed by De Vogel (1980) to refer to leaf-like photosynthetic cotyledons based on a recent finding that these structures are of different developmental origin as compared to the food storing and haustorial types.

Leaves rolled (convolute) in the bud, edges of leaf buds definitely overlapping 7

7 Leaf sheaths glabrous ..... 6. *Centhoteca latifolia* (L.) Trin. (kaua-kauayanan)

Leat sheaths hairy 8

8 Ligules fringed with hairs, truncate, the hairs 1/2 the entire length ....

..... 7. *Setaria palmifolia* (Koenig) stapf (agusais)

Ligules not as above 9

9 Leaf blades scabrous on both surfaces 10

Leaf blades scabrous on upper surface only 11

10 Nodes bearded ... 8. *Cyrtococcum oxyphyllum* (Steud) Stapf (banig-usa)

Nodes not bearded ... 9. *Digitaria ciliaris* (Retz.) Koel. (crabgrass)

11 Leaf sheaths tuberculate-hispid or scabrous, green, ovate in section

..... 10. *Rottboellia cochinchinensis* (Lour.) Clayton (agingay)

Leaf sheaths glabrous or with few hairs confined at the margins, not tuberculate-hispid or scabrous, compressed, with tinge of purple .....

..... 11. *Paspalum conjugatum* Berg. (lau-lau)

SEEDLING DESCRIPTION

Cyperaceae

1. *Cyperus kyllingia* Endl.

Seedlings hypogeal. Taproots absent, the adventitious roots white to dirty white, flexuous. Culms triangular in cross section. Cotyledon 1, non-emergent. Mesocotyl well developed, ca 2.5 mm long. Leaf sheaths membranous, closed, with streaks of pink and purple, glabrous. Leaf blades glabrous, 2.5 cm long, 1 mm wide, margins scabrous, collar glabrous. Succeeding leaves larger, to 10 x 1.2 cm.

Figure 2. ***Cyperus kyllingia*****: 1. habit; 2. portion of culm with sheath at base; 3. flower; same glumes excised; 5. achene; 6a. seedling at second leaf; 6b and 6c. older seedlings.**

2. *Cyperus michelianus* (L.) Link

Seedlings hypogeal. Taproots absent, the adventitious roots straight to slightly flexuous, dirty white to brownish. Culms triangular. Cotyledon 1, non-emergent. Mesocotyl weakly developed. Leaf-sheaths membranous, closed, whitish, with streaks of pink or purple, glabrous. Leaf blades glabrous, the first 5 cm long, 0.4-0.5 mm wide, keeled in cross section, apex pointed. Succeeding leaves longer, the fourth 15 cm long, 0.9 mm wide.

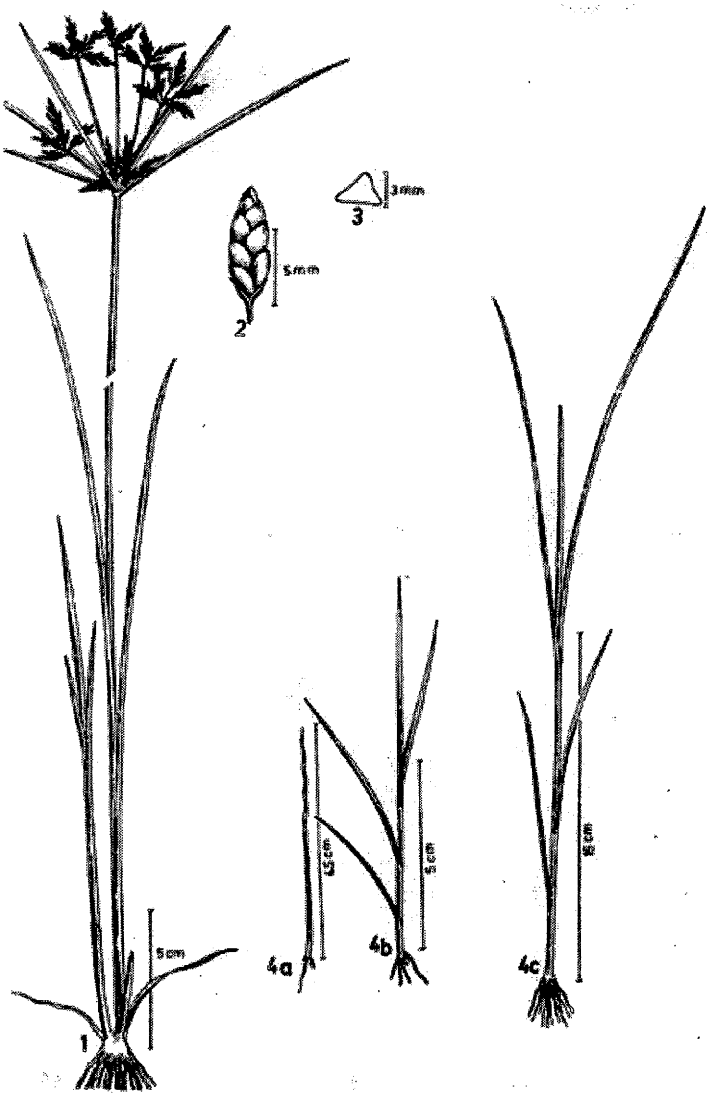

Figure 3. *Cyperus michelianus*: 1. habit; 2. spikelet; 3. cross section of culm; 4a. seedling at first stage; 4b & 4c. older seedlings.

Commelinaceae

3. *Dictyospermum vitiense* (Seen) J.K.Morton

Seedlings hypogeal, bearded at union of leaf blade and outer margin of leaf sheath. Taproots absent, adventitious roots white to dirty white, slightly flexuous, with few flexuous branch roots. Cotyledon 1, non-emergent. Stem circular in cross-section, erect. Mesocotyl undeveloped. Leaf sheaths hairy on the outer side, light green to whitish, the base tinged with purple, open, membranous. Leaf blades ovate, green above, light green below, scabrous on both surfaces; margins entire, ciliate; apex acute; base more or less truncate, nerves almost distinct. First leaf 1.5 x 1.0 cm.

Figure 4. *Dictyospermum vitiense*: 1. habit; 2. fruit; 3a & 3b. seedlings at first stage; 3c & 3d. older seedlings.

4. *Aclisia secundiflora* (Bl.) Bakh

Seedlings hypogeal. Taproots absent, the primary roots creamy to brownish, replaced by wiry seminal roots, slightly flexuous, with numerous creamy, flexuous branch roots. Stems circular in cross section, erect. Cotyledon 1, non-emergent. Mesocotyl undeveloped. Leaf sheaths hairy on the outer side, glabrous on the inner side, circular in cross section, light green to whitish green, membranous. Leaf blades flat, elliptic, green above, light green below, scabrous above, apex acute, base obtuse, margins ciliate, entire, nerves distinct. First leaf about 0.6 x 0.4 cm. Succeeding leaves larger, in the fourth, ca 2.5 x 0.6 cm.

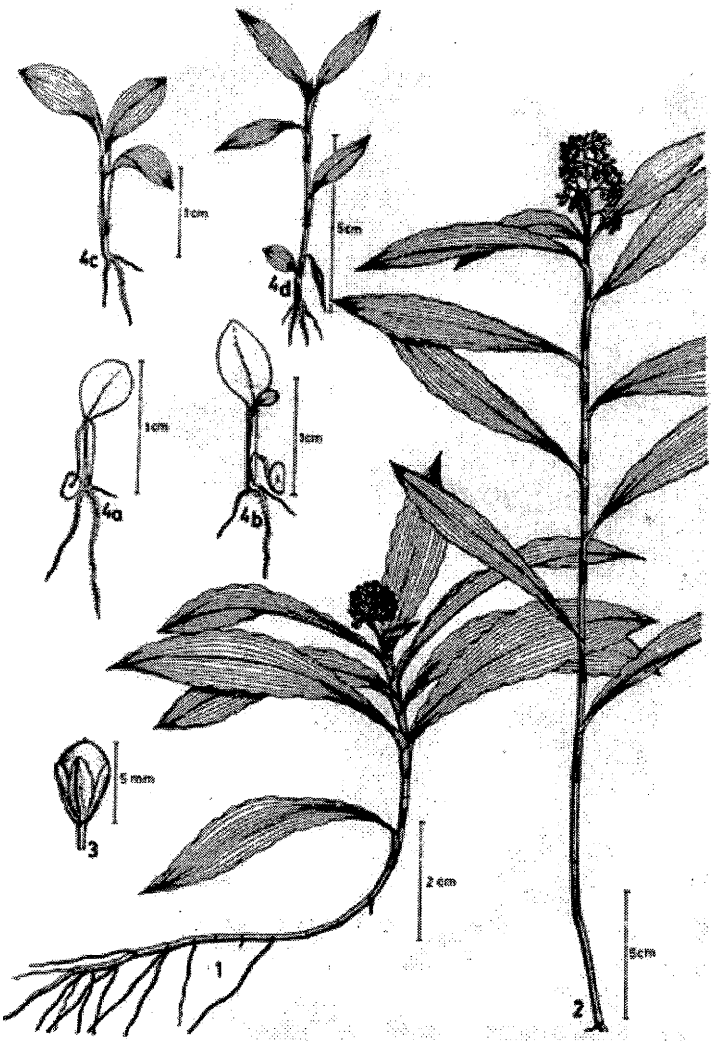

Figure 5. *Aclisia secundiflora*: 1. habit; 2. flower branch; 3. mature fruit; 4a. & 4b. seedlings at first stage; 4c & 4d. older seedlings.

Graminae (Poaceae)

5. *Eleusine indica* (L.) Gaertn.

Seedlings hypogeal. Taproots absent, adventitious roots white to dirty white, slightly flexuous or straight. Vernation folded. Mesocotyl weakly developed. Cotyledon 1, non-emergent. Leaf sheaths glabrous, flat or keeled in cross section, green to light green, in the first sheath 4 mm long, in the 3rd 1.3 cm long. Ligules membranous, glabrous, truncate with jagged edge, in the 2nd leaf 0.7 mm long. Collars glabrous, with few long, soft hairs at base, in the first leaf 1.5 without ciliate hairs; nerves almost distinct.

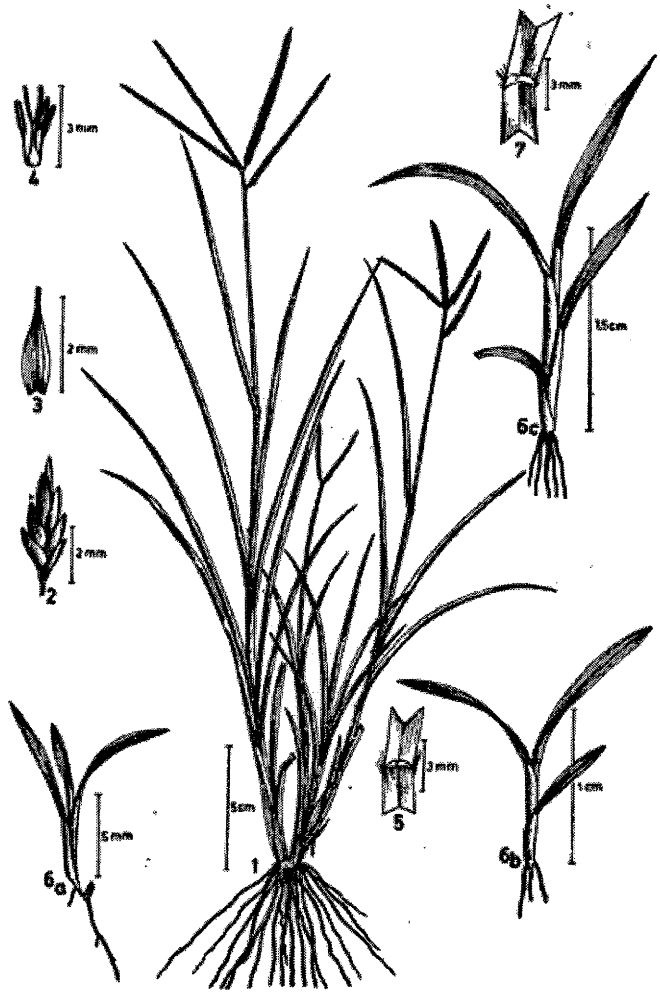

Figure 6. *Eleusine indica:* 1. habit; 2. spikelet; 3. bract; 4. flower; 5. portion of sheath and blade to show ligule of adult plant; 6a & 6b. seedling at first stage; 6c. older seedling; 7. portion of sheath and blade to show ligule of seedling.

6. *Centhoteca latifolia* (L.) Trin.

Seedlings hypogeal. Taproots absent,the adventitious roots white to dirty white, straight to slightly flexuous, culms erect, glabrous at nodes, circular in cross section. Mesocotyl well developed. Cotyledon 1, non-emergent. Leaf sheaths glabrous, whitish near base, green near collar, oval or circular in cross section, in the first ca 0.4 cm long, longer in succeeding sheaths. Ligule membranous, truncate, entire, glabrous, sometimes split. Collars glabrous. Blades with few hairs at base, otherwise glabrous, in the 7th ca 5.5 cm long, 1.5 cm wide; margins finely serrate or ciliate; nerves almost distinct. Vernation rolled (convolute).

Figure 7. *Centhoteca latifolia:* 1. habit; 2. portion of sheath and blade to show ligule of mature plant; 3 & 4. immature spikelets; 5,6,7,8. glumes; 9a. seedling at third leaf; 9b & 9c. older seedlings; 10. portion of sheath and blade to show ligule of seedling.

7. *Setaria palmifolia* (Koenig) Stapf

Seedlings hypogeal. Taproots absent, the adventitious roots white to off-white, slightly flexuous, 0.5-1 cm long in the first stage, with numerous short hairs. Vernation rolled. Mesocotyl ca 2 mm in the first stage, light green to purplish, terete, glabrous. Cotyledon 1, non-emergent. Leaf sheaths glabrous except for the long pilose hairs confined along the margins, pink or purplish in first stage, more or less circular in cross section. Ligule membranous, truncate, margins hairy, the hairs ca 1/2 the entire length,0.4 mm long in the 3rd leaf. Collars hairy. Blades linear, scabrous on both surfaces, green, 4-4.5 cm long, 0.5-0.6 cm wide in 3rd leaf, nerves distinct, palm-like.

Figure 8. *Setaria palmifolia:* 1. habit; 2. portion of sheath and blade to show ligule of mature plant; 3. spikelet; 4a & 4b. seedlings at first stage; 4c. older seedling; 5. portion of sheath and blade to show ligule of seedling.

8. *Cyrtococcum oxyphyllum* (Steud.) Stapf

Seedlings hypogeal. Taproots absent, the adventitious roots white to dirty white, straight or slightly flexuous, with few to numerous flexuous lateral roots. Culms erect to prostrate with age, circular in cross section, bearded at nodes. Vernation rolled, mesocotyl elongating to ca 3 mm long, straight or slightly flexuous, cotyledon 1, non-emergent. Leaf sheaths with long soft hairs specially near the base and collar, circular in cross section, sometimes tinged with purple, otherwise green to light green, the first ca. 0.5 cm long. Ligules membranous, truncate, entire, free of hair, in the 3rd leaf 0.6 mm, longer in succeeding leaves. Collars with long soft hairs. Blades elliptic in the first 5 leaves, becoming linear in succeeding leaves, flat in cross section, sparsely covered with short pilose hairs on both surfaces, green above, light green below; base inequilateral; hairy; margins ciliate; nerves almost distinct.

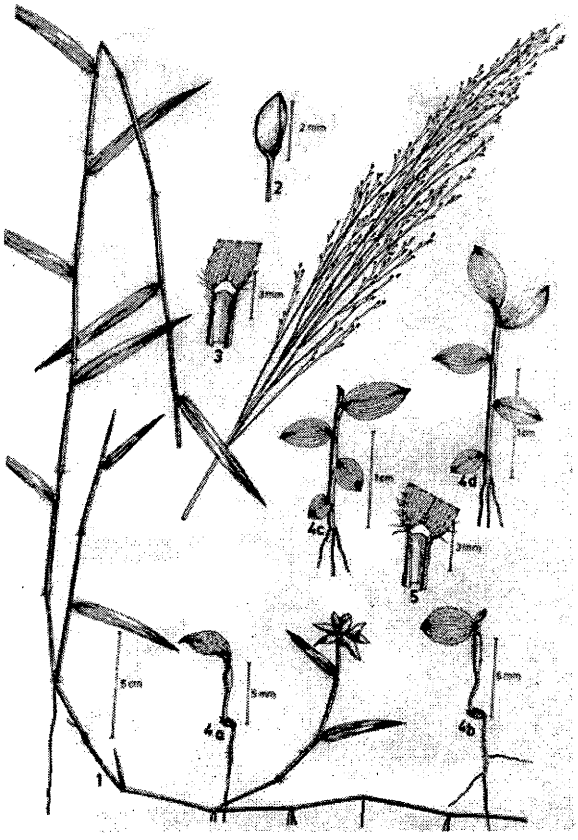

Figure 9. *Cycrtoccocum oxyphyllum:* 1. habit; 2. immature seed; 3. portion of sheath and blade to show ligule of mature plant; 4a & 4b. seedlings at first stage; 4c & 4d. older seedlings; 5. portion of sheath and blade to show ligule of seedling.

9. *Digitaria ciliaris* (Retz.) Koel.

Seedling hypogeal. Taproots absent,the adventitious roots white to dirty white, flexuous, with few flexuous lateral roots at first stage. Vernation rolled. Mesocotyl elongating to ca 2 mm long, glabrous, straight. Cotyledon 1, non-emergent. Leaf sheaths oval in cross section, green to light green, with scattered pilose hairs, the hairs somewhat dense near the base. Ligules membranous, collar shaped, margin entire, in the first leaf, becoming toothed in succeeding leaves, in the second leaf 2 mm long, 1 mm in the first. Collars with few pilose hairs, otherwise glabrous. Blades scabrous on both surfaces, flat, in first 2 leaves 2-3 cm long, 2-3 cm wide, linear or elliptic, nerves indistinct except for the light green midrib.

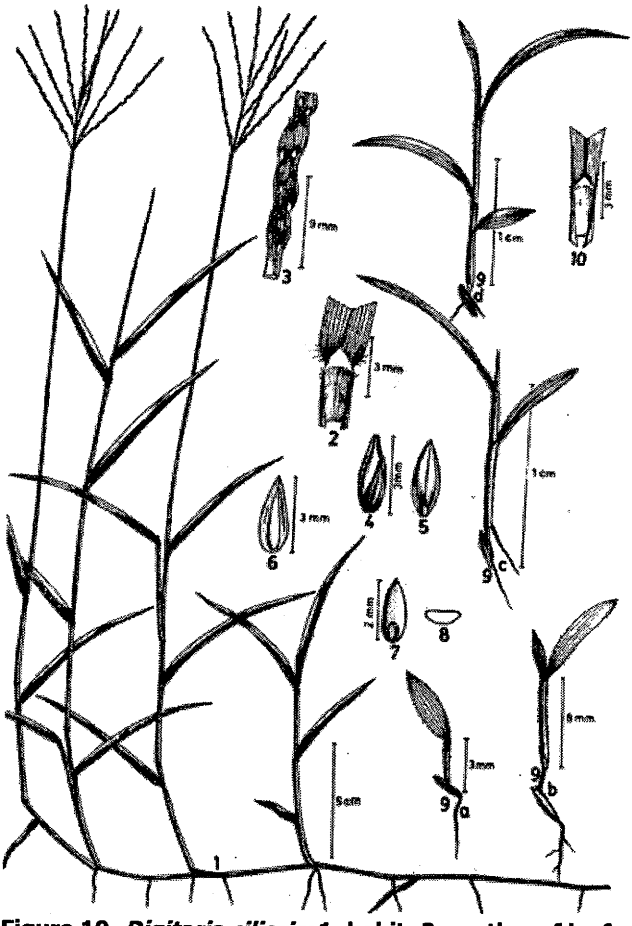

Figure 10. *Digitaria ciliaris:* 1. habit; 2. portion of leaf sheath to show ligule of mature plant; 3. portion of spikelet, enlarged; 4 & 5. spikelet; 6. bract; 7. grain; 8. same, cross section stage; 9a. seedling at first stage; 9b & 9c. seedling at second stage; 9d. older seedling; 10. portion of sheath and blade to show ligule of seedling.

10. *Rottboellia cochinchinensis* (Lour.) Clayton

Seedlings hypogeal. Taproots absent, the adventitious roots flexuous, off-white, 2.5 cm long in first stage. Culms circular in cross section, erect, green to light green. Vernation rolled. Mesocotyl well developed, ca 2-3 cm in the first stage, purplish, straight. Cotyledon 1, non-emergent. Leaf sheaths with short tuberculate-hispid hairs especially near the collars, green to light green, split, oval to circular in cross section, 0.9 cm long in first leaf, longer in succeeding leaves. Ligules membranous, free of hair, truncate, notched or split at edge, 0.9 mm in 2nd leaf. Collars with hispid hairs. Blades linear, scabrous above, glabrous below, both surfaces green to light green, hairy especially at base, 3.3 cm long, 0.5 cm wide in first leaf, longer in succeeding leaves, flat or slightly keeled in section, margins serrulate, nerves almost distinct, the midnerves whitish.

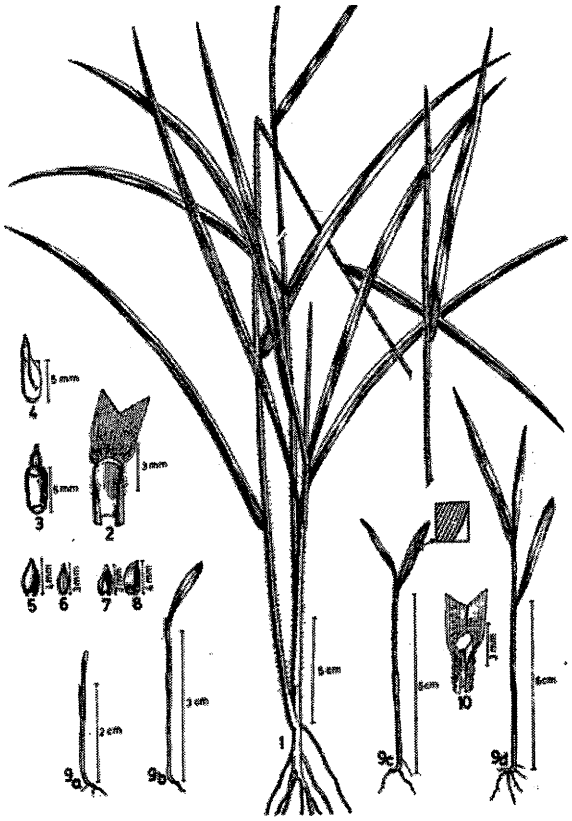

Figure 11. *Rottboellia conchinchinensis:* 1. habit; 2. portion of leaf sheath to show ligule of mature plant; 3 & 4. joints with pedicel and sessile spikelet; 7 & 8. lower and upper glumes, both of pedicellate spikelet; 9a & 9b. seedlings at first stage; 9c & 9d. older seedlings; 10. portion of sheath and blade to show ligule of seedling.

11. *Paspalum conjugatum* Berg.

Seedlings hypogeal. Taproots absent, the adventitious roots white or dirty white, flexuous, with few to numerous lateral roots. Culms green to purplish, erect in the first stage, becoming prostrate with age, flat. Vernation rolled. Mesocotyl weakly developed. Leaf sheaths split, compressed, keeled, glabrous except for the hairs at base and margins,0.6-0.7 mm long in the first sheath, green to purplish. Ligules membranous, ca 0.5-1 mm long in 3rd leaf, truncate, entire or with jagged edge, free of hair. Collars hairy. Blades glabrous or sparingly hairy, linear, flat in cross section, green above, with tinge of purple below, 0.2 cm long, 2 mm wide in first leaf; margins ciliate, the hairs arranged in 2 ranks.

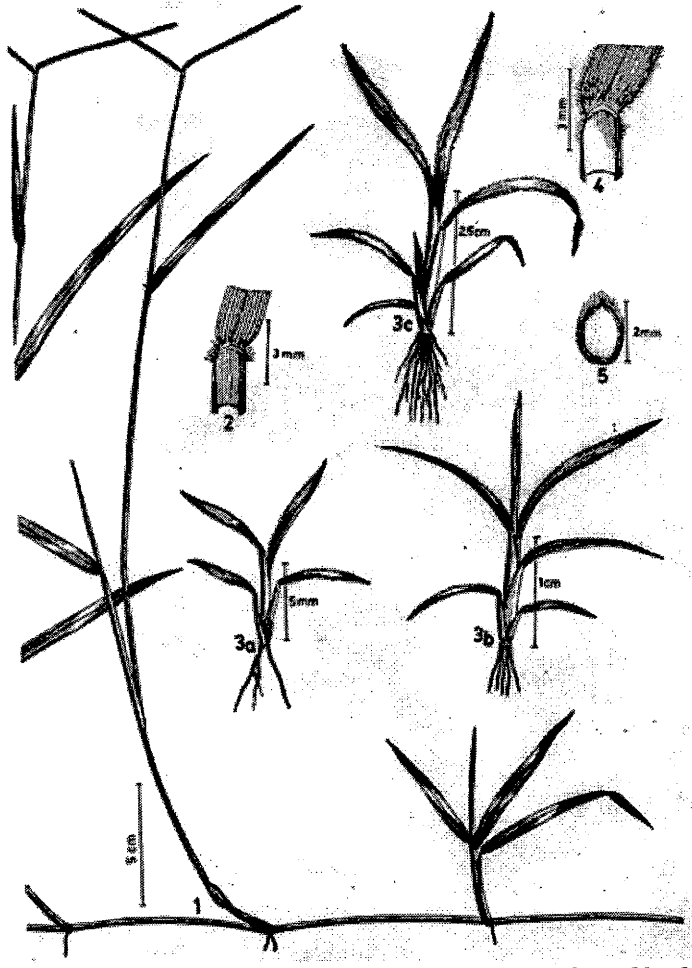

Figure 12. *Paspalum conjugatum:* 1. habit; 2. portion of leaf sheath to show ligule of mature plant; 3a & 3b. seedlings at second stage; 3c. older seedling; 4. portion of sheath and blade to show ligule of seedling; 5. spikelet.

SUMMARY AND CONCLUSION

Although the number of species examined and presented in this report is very limited, the results nevertheless showed that there are diagnostic morphological characters in a monocot seedling which can be utilized for their correct identification and classification.

Hypogeal germination is a distinctive feature of monocot seedlings. Several morphological characters are considered diagnostic including the mesocotyl, culm/or stem, cotyledonary stalk and leaf. In general, the leaf offers many important clues for identification. However, identification usually involves combination of as many characters as possible. In many instances, a single character is insufficient to ascertain correct specific identification.

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APPENDIX A.

Glossary of botanical terms used in the description of seedlings and key to identification of species

Ciliate - bearing a fringe of hairs on the margins

Collar - in monocot leaf, the junction between the leaf blade and leaf-sheath.

Convolute, rolled vernation - said of leaf envelopes in the bud when the edge overlaps the next part while the other edge is overlapped by a preceding part; the margins overlapping

Cotyledon - the primary leaf in the embryo of seedling in the Angiosperms

Elliptical, elliptic - broadest near the middle with the sides tapering equally to a point; has the form of an ellipse

Entire - with a continuous margin; not in any way indented (may or may not be hairy or ciliate)

Epigeal, epigeous - above the soil level; for seedlings, referring to the position opf the cotyledons after germination

Flexuous - having a more or less zigzag or wavy form

Folded vernation - in contrast to convolute vernation, said of leaf envelopes in the bud when the edges do not overlap

Glabrous - free of hair or pubescence

Hispid - provided with stiff or bristly hairs

Hypogeal, hypogeous - the cotyledon stays below the soil level after germination typical of the monocot

Keeled - ridged like the bottom of a boat

Leaf sheath - in monocot leaf, the part of the leaf enveloping the stem

Ligule - an outgrowth (either a membrane or a fringe of hairs) on the inner (upper, adaxial) surface at the junction between the sheath and blade in the leaves of grasses

Linear - long and narrow, the sides parallel or nearly so, as the blades of most grasses and sedges

Membrane - a thin, transparent tissue

Mesocotyl - internode between the scutellar node and the coleoptile in the embryo and seedling of grasses and sedges

Oval - broadly elliptical

Ovate - egg-shaped, broadest near the base

Pilose - shaggy with soft hairs

Primary root - equals taproot

Prostrate - a general term for lying flat on the ground

Scabrous - rough to touch

Serrate - awl-shaped; said of a margin when saw-toothed, with the teeth pointing forward

Taproot - the first, or primary root of a plant forming a direct continuation of the radicle of the embryo

Truncate - ending abruptly as if cut off

Tuberculate - with tubercles

Vernation - the disposition or arrangement of leaves in the bud as seen from a cross section obtained by cutting across the outer sheath at the point just below the collar of the last fully emerged leaf

Role of Nonpituitary Gonadotrophins on Reproductive Activities in Female Lizard *Calotes versicolor*

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ABSTRACT

The nonpituitary gonadotrophins both P M S G and h C G induce significant changes in the ovarian weight, number and diameter of the follicles. The nuclear diameter of the oocyte is also increased. The granulosa layer of the follicles becomes thicker with hypertrophied cells. There is a significant increase in the ovarian cholesterol level which indicates that the cholesterol is utilized for steroidogenesis. The weight of the oviduct and its protein content increases significantly which supports the production of steroid hormones from the ovary. The combined gonadotrophin treatment is more effective than the individual gonadotrophin treatment. However, these activities towards the elevated side could not be comparable to those of breeding season as still higher activities are seen during breeding season.

INTRODUCTION

It is well established that the mammalian pituitary gonadotrophins both Follicle stimulating hormone (FSH) and Luteinizing hormone (LH) stimulate spermatogenesis in reptiles (Licht and Pearson, 1969; Reddy and Prasad, 1970 a and b ; Angelini et al, 1978; Gaitonde and Gouder, 1985). The studies on the action of FSH and LH on ***Anolis carolinensis*** (Licht and Pearson, 1969), ***Hemidactylus flaviviridis*** (Reddy and Prasad, 1970a and b), ***Uromastix hardwicki*** (Jalali et al, 1975), and ***Calotes versicolor*** (Gaitonde and Gouder, 1985) indicates that the reptilian spermatogenesis and steroid harmone production could be regulated by mammalian pituitary gonadotrophins FSH and LH.

Eyeson (1971) has reported that FSH is essential for the maintenance of spermatogenesis and LH selectively acts upon the Leydig cells in the lizard ***Agma agma***, but in the lizard ***Lacerta sicula*** mammalian FSH and LH stimulate both spermatogenesis and steroidogenesis

during nonbreeding season and LH specifically acts on later stages of spermatogenesis (Angelini et al. 1978). However, the studies on the effect of gonadotrophins on gonadal activities in female reptiles are scanty. In reptiles the release of pituitary gonadotrophins is dependent on environmental factors such as rain fall, humidity, temperature, photoperiod, etc.

The lizard C. versicolor from Hyderbad Karnatak region of India breeds from May to August and its gonads remain in the regression phase from September to January and in the regeneration phase from February to April (Hallikhed, 1990). In the present investigation an attempt to stimulate the reproductive activities like ovarian growth, follicular formation, ovulation, steroidogenesis and oviductal response during the nonbreeding season was made and compared with that of normal breeding activities of C. versicolor.

MATERIAL AND METHODS

Adult female C. versicolor during the breeding and nonbreeding seasons were collected from the Hyderabad Karnataka region. The specimens weighing 40-50gms and with snout vent length 14-15cms were selected for experimentation. The animals were acclimatized to the laboratory conditions for 5 days and fed with live cockroaches and water **ad libitum**.

Experiment I

Six animals collected during the breeding season received 5 injections of 0.2ml of 0.9% isotonic saline solution every alternate day.

Experiment II

The animals during the regeneration and regression phases of the nonbreeding season were divided into four groups of six animals per group. **Group 1**. For the female lizards received 5 injections of 0.2ml saline on every alternate day and served as the control. **Group 2**. Lizard received 5 injections of 5 IU P M S G (Folligon, Intervet, International, B.V. Boxmere, Holland) in 0.2ml isotonic saline at every alternate day. **Group 3**. Lizard received 5 injections of 5 IU h C G (Chorulon, Intervet, International, B.V.Boxmere, Holland) in 0.2ml isotonic saline at every other day. **Group 4**. Lizard received 5 injections of 5 IU PMSG + hCG in 0.2ml isotonic saline at every other day. At autopsy on the 11th day after treatment, 48 hours after the last injection, the ovary and oviduct were dissected out and weighed to the nearest mg. The ovarian cholesterol content from one ovary of each animal was estimated using Libermann and Burchard method modified by Peters and Van slyke (1946). Similarly, the protein content was estimated with Lowry's method (1951). The other

ovary and oviduct of each animal were fixed in Bouin's fluid, embedded in paraffin, sectioned with 5m thickness and stained with Ehrlich's haematoxyline-eosin. The number of follicles were counted from randomly chosen 20 sections of each group. The histometric measurements like diameters of the follicles, oocyte nucleus and thickness of granulosa were measured by using ocular and stage micrometer. The statistical analysis were carried out by using Student's 't' test and 'p' value <0.05 were considered as significant.

RESULTS

Changes in the ovarian weight (Table-1)

The ovarian weight during both regeneration and regression phases significantly decreased than that of breeding season (P<0.001). But the 5 IU gonadotrophin treatment either separately or in combination increased the ovarian weight significantly (P<0.001). However, this increase in ovarian weight at the 5 IU dose level was comparatively less when compared to that of the ovary during the breeding season.

Table 1. Comparative changes in the ovarian weights and total cholesterol contents of the breeding and nonbreeding (regeneration and regression phases) seasons after gonadrophins (PMSG & hCG) administration in *C. versicolor*.

Treatment	Wt. of Ovary mg/100gm body wt.	Cholesterol content µg/100mg ovary
Breeding Season		
Saline soln.	562.1 ± 32.2	589.2 ± 16.2
Nonbreeding Season (Regeneration Phase)		
Saline soln.	10.2 ± 3.3	1904.0 ± 188.6
PMSG	142.8 ± 20.6*	254.5 ± 42.4*
hCG	203.7 ± 22.7*	300.4 ± 33.4*
PMSG + hCG	257.2 ± 34.8*	215.6 ± 39.5+
NonBreeding Season (Regression Phase)		
Saline soln.	11.7 ± 1.6	1250.0 ± 64.5
PMSG	147.7 ± 9.8*	376.4 ± 49.5*
hCG	131.5 ± 9.4*	302.0 ± 29.3*
PMSG + hCG	140.6 ± 8.6*	191.2 ± 28.6*

M ± S.E = Mean ± Standard Error
* P < 0.001 when compared to respected saline treated controls
P < 0.001 when any gonadotrophin treated group was compared to that of saline treated group of breeding season.

Ovarian cholesterol (Table - 1)

The ovarian cholesterol level during the nonbreeding season was significantly higher compared to that of the ovary during the breeding season is high ($P<0.001$). However, it is decreased by about 4-6 folds during regeneration and the regression phases after gonadotrophin treatment. The cholesterol level dropped significantly ($P <0.001$) after gonadotrophin treatment at both ovarian phases. The decrease in cholesterol level was significant ($P <0.001$) when compared to that of the ovary during breeding season.

Follicular Changes (Table -2)

There was a significant ($P <0.001$) reduction in the number of follicles at the regeneration and regression phases when compared to that of the ovary during the breeding season. Though the gonadotrophin treatment either separately or in combination had increased the number of follicles significantly ($P <0.001$), it was remarkably less when compared to that of the ovary during the breeding season. Gonadotrophin treatment during the regeneration and regression phases increased the thickness of the granulosa layer, follicular diameter and nuclear diameter of the oocyte significantly ($P <0.001$), when compared to the measurement of the same structure of saline solution the controls. The combined gonadotrophin treatment was more effective than the single gonadotrophin treatment.

Table 2. Changes in the number of follicles, thickness of granulosa layer, follicular diameter and nuclear diameter of occyte between breeding and nonbreeding (regeneration and regression phases) seasons after gonadotrophin (PMSG and hCG) treatment in *C. versicolor*.

Treatment	Number of Follicles	Thickness of Granulosa (μm)	Follicular Diameter (μm)	Nuclear Diameter of occyte (μm)
Breeding Season				
Saline soln.	15.21 ± 0.6	212.6 ± 10.6	506.8 ± 30.3	91.62 ± 4.9
Nonbreeding Season (Regeneration Phase)				
Saline soln.	4.80 ± 0.4	91.6 ± 5.5	489.4 ± 19.2	57.8 ± 4.5
PMSG	7.55 ± 0.8*	128.9 ± 5.5*	562.2 ± 24.4*	62.2 ± 3.4*
hCG	8.55 ± 0.3*	131.2 ± 3.1*	639.9 ± 28.9*	84.9 ± 2.6*
PMSG + hCG	9.96 ± 0.5*	126.1 ± 7.2*	226.1 ± 26.5*	94.9 ± 3.7*
NonBreeding Season (Regression Phase)				
Saline soln.	3.70 ± 0.3	91.6 ± 5.6	188.4 ± 10.5	43.3 ± 3.9
PMSG	7.75 ± 0.7*	162.7 ± 5.6*	319.4 ± 19.3*	74.9 ± 3.1*
hCG	9.35 ± 0.5*	180.3 ± 9.4*	392.8 ± 21.0*	77.7 ± 4.6*
PMSG + hCG	9.98 ± 0.5*	190.5 ± 9.8*	439.4 ± 3.1*	86.6 ± 5.5*

M ± S.E = Mean ± Standard Error

* $P < 0.001$ when compared to respective saline treated controls.

Changes in the oviduct (Table -3)

The growth of the oviduct and its protein content reflect the level of ovarian activity. The weight of the oviduct during the nonbreeding season was significantly ($P < 0.001$) lesser then that of the breeding season. The protein content of the oviduct also significantly ($P < 0.001$) lesser. After gonadotrophin treatment there was an increase in the weight of the oviduct and its protein content during both regeneration and regression phases.

Table 3. Changes in the weight of oviduct and its protein content during breeding and nonbreeding (regeneration and regression phases) seasons after gonadotrophin treatment in *C. versicolor*.

Treatment	Wt. of Ovary mg/100gm body wt.	Cholesterol content µg/100mg ovary
Breeding Season		
Saline soln.	650.8 ± 60.2	3.48 ± 0.04
Nonbreeding Season (Regeneration Phase)		
Saline soln.	159.8 ± 7.8	1.62 ± 0.02
PMSG	233.0 ± 41.9*	2.67 ± 0.00*
hCG	359.2 ± 11.4*	2.15 ± 0.01*
PMSG + hCG	366.3 ± 52.4*	2.72 ± 0.01*
NonBreeding Season (Regression Phase)		
Saline soln.	300.3 ± 19.2	2.22 ± 0.01
PMSG	541.8 ± 66.2*	6.94 ± 0.04*
hCG	504.8 ± 52.3*	4.44 ± 0.20*
PMSG + hCG	521.3 ± 55.9*	6.00 ± 0.23*

M ± S.E = Mean ± Standard Error
*P < 0.001 when compared to respective saline treated groups
P < 0.001 when any gonadotrophin treated group compared with that of saline treated group of breeding season.

DISCUSSION

Previous studies on the effects of pituitary gonadotrophins (FSH & LH) on the reproductive activities of ***Anolis carolinensis***, ***Hemiductylus flaviviridis*** and ***Calotes versicolor*** have been limited to the male reproduction only (Licht and Pearson 1969; Reddy and Prasad 1970, a & b; Gaitonde and Gouder 1985). These studies indicate that FSH is more potent than LH, though both the hormones are capable of stimulating the spermatogenesis and steroidogenesis, but such studies are limited in female

reptiles, may be because of the timely events of reproductive activities. In the present investigation, the exogenous nonpituitary gonadotrophins administered during the nonbreeding season have increased the ovarian activities several times. However, this can not be equated to the similar ovarian activity that takes place during the breeding season. The low level of ovarian cholesterol has been utilized for steroid hormone production. The fact that no corpora lutea has been observed in the treated ovary and no egg is present in the oviduct indicates the absence of ovulation.

These observations indicate that optimal temperature, photoperiod and moisture are also necessary for the completion of ovarian activities along with the availability of gonadotrophins in C. versicolor.

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Screening of Some Essential Oils Against Dermatophytes

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ABSTRACT

The essential oils of cinnamomum tamala and Citrus maxima exhibited absolute inhibition of mycelial growth on deramatophtes viz. Trichphyton mentagrophytes and Microsporum audouini. The minimum inhibitory concentration (MIC) of both the oils was found to be 500 ppm at whci they exhibited fungistatic nature, broad mycotic spectrum and long shelf life. Their fungitoxic properties remained thermostable in nature up to 100°C. Besides, the oils showed superiority in fungitoxic potency over some prevalent synthetic antifungal drugs

INTRODUCTION

Green plants particularly of the tropical flora are reservoirs of various biologically active substances (Sbragia, 1975). In recent years several products of higher plants have been standardized as potential biotoxicants against different plant pathogenic fungi. However, there is little attention paid on the activity of such products against human pathogenic fungi causing dermatomycosis (Dubey and Mishra, 1990; Kishore et al. 1993). In contrast to different synthetic which possess severe side effects (stevens et al. 1976; Paterson et al. 1980; Symoens et al. 1980), the plant products which are in use in different forms in Ayurvedic system of medicine since long, would have least mammalian toxicity . In the present investigation the essential oils of some higher plants have been isolated and bioassayed against the test dermatophytes viz. *Trichophyton mentagrophtes* and *Microsporum audouinii* causing ringworm diseases to animals and human beings. The essential oils showing absolute activity have been subjected to detailed investigations.

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MATERIAL AND METHODS

Leaves of ten angiospermic taxa viz *Aegle marmelos* Linn., *Amomum subulatum* Roxb., *Callistemon lanceolatus* (D.C.), *Cinnamomum camphora* Linn., *C. tamala* Nees., *Citrus maxima* (Burm.), *C. reticulata* Blanco, *C. sinensis* (Linn), *Hyptis suaveolens* (L) Poir and *Murraya koenigii* (Linn.) were collected from Ayurvedic garden, Banaras Hindu University, Varanasi, washed in distilled water and were subjected to hydrodistillation so as to collect the essential oils by the methods suggested by Guenther (1972). The oils were tested for their fungitoxicity against *T. mentagrophyes* and *M. audounii* by the poisoned food technique of Groverand Moore (1962). 0.01 ml of the oils were dissolved separately in 0.05 ml of acetone in presterilized pertridishes and mixed thoroughly with 9.5 ml of sabouraud dextrose agar medium to obtain concentrations of 1000 ppm. Control sets were prepared similarly using sterile water in place of the oils. Fungal discs of 5 mm diameter cut from the periphery of 7 day-old culture of the test dermatophytes were placed aseptically in centre of each petridish separately and the dishes were incubated at 28±1oC for 6 days. The diameters of fungal colonies of treatment and control seats were measured on the 7th day. The percent mycelial inhibition was calculated by the following formula:

$$\text{Percent inhibition of mycelial growth} = \frac{d_c - d_t}{Dc} \times 100$$

Where,

Dc=Average fungal colony diameter in control set
Dt=Average fungal colony diameter in treatment set

The oils of *Cinnamomum tamala* and *Citrus maxima* exhibited strong fungitoxicity against both the dermatophytes and were thus subjected to further studies. The minimum inhibitory concentration (MIC) of the oils were determined by the usual poisoned food technique. For this different graded concentrations of the oils viz. 50, 100, 200, 300, 400 and 500ppm were prepared with respect to sabouraud dextrose agar medium and were inoculated separately with test dermatophytes. The percent inhibition of growth of the dermatophytes at each concentration was noted in comparison to control set. The fungistatic/fungicidal nature of the effective oils were tested by the technique adopted by Thompson (1989). The fungal dics whose growth was completely inhibited were reinoculated to fresh sabouraud dextrose agar medium after washing in distilled water and their revival of growth was observed . Mycotoxic spectrum of the oil also observed by the usual poisoned food technique against eight human pathogenic fungi. The shelf life of the oils and effect of temperature on their toxicity were evaluated following Mishra et al. (1930). The

antifungal efficacy of the oils was compared with some prevalent synthetic antifungal agents viz. Clotrimazole, benzoic acid, griseofulvin, kitaconazole, nystatin and salicylic acid by the usual poisoned food technique.

RESULTS

Among ten essential oils of angiospermic taxa tested, only the oils leaves of *Cinnamomum tamala* and *Citrus maxima* exhibited absolute toxicity against both the dermatophytes viz *T. mentagrophytes* and *M. audounii*. The rest of the oils showed either poor or moderate activity (Table 1).

Table 1. Screening of essential oils against *Microsporum audounii* and *Trichophyton mentagrophytes* at 1000 ppm.

Essential oils	Percent inhibition of mycelial growth on test fungi	
	Microsporum audouinii	*Trichophyton mentagrophytes*
Aegle marmelos (Linn.)	7	39
Amomum subulatum (Roxb)	70	88
Callistemon lanceolatus (DC)	61	52
Cinnamomum camphora (Linn.)	82	80
C. tamala Nees.	100	100
Citrus maxima (Burm)	100	100
C. reticulata Blanco	28	30
C. sinensis (Linn.)	40	22
Hyptis suaveolensis (L) Poir	58	66
Murraya koenigii (Linn.)	31	30

The minimum inhibitory concentration of these oils for complete inhibition of the dermatophytes was found to be 500 ppm. The oil of *Cinamomum tamala* was fungistatic in nature up to 700ppm against both the test dermatophytes and turned fungicidal at 800ppm. The *Citrus maxima* oil was fungistatic in nature up to 800 ppm against both the test fungi and turned fungicidal at 900 ppm (Table 2). The essential oil of *Cinnamomum*

Table 2. Minimum inhibitory concentration (MIC) of *Cinnamomum tamala* and *Citrus maxima* oils and their nature of toxicity.

Concentration (in ppm)	Percent inhibition of mycelial growth on test fungi			
	Cinnamomum tamala		*Citrus maxima*	
	M. A.	T. M.	M. A.	T. M.
50	9	8	18	17
100	22	31	30	22
200	71	46	56	43
300	45	87	72	68
400	90	90	80	96
500	100^{++}	100^{++}	100^{++}	100^{++}
600	100^{++}	100^{++}	100^{++}	100^{++}
700	100^{++}	100^{++}	100^{++}	100^{++}
800	100^{+}	100^{+}	100^{++}	100^{++}
900	100^{+}	100^{+}	100^{+}	100^{+}
1000	100^{+}	100^{+}	100^{+}	100^{+}

M. A. = Microsporum audouinii, T. M. = Trichophyton mentagrophytes, ++ = Fungistatic
+ = fungicidal

tamala inhibited the mycelial growth of two and seven fungi at 500 and 1000 ppm respectively. Of eight human pathogenic fungi tested, only *Aspergillus terreus* was found to be resistant against the oil (Table 3). The

Table 3. Fungitoxic spectrum of *Cinnamomum tamala* oil at various concentrations against human pathogenic fungi.

Dermatophytic fungi	Percent inhibition of mycelial growth (Concentration (ppm))		
	250	500	1000
Aspergillus flavus	0	50	100
Aspergillus fumigatus	30	80	100
Aspergillus niger	0	45	100
Aspergillus terreus	21	70	80
Epidermatophyton floccosum	39	80	100
Microsporum gypseum	20	100	100
Trichophyton rubrum	50	82	100
Trichophyton violaceum	55	100	100

essential oil of *Citrus maxima* completely inhited three and fungi at 500 and 1000 ppm respectively. *Aspergillus flavus*, *A. fumigatus*, *A. niger*, and *A. terreus* were found to be resistant up to 1000 ppm (Table 4). Thus, the

Table 4. Fungitoxic spectrum of *Citrus maxima* oil at various concentrations against human pathogenic fungi.

Dermatophytic fungi	Percent inhibition of mycelial growth (Concentration (ppm))		
	250	500	1000
Aspergillus flavus	0	18	80
Aspergillus fumigatus	30	40	48
Aspergillus niger	0	20	60
Aspergillus terreus	8	20	40
Epidermatophyton floccosum	30	100	100
Microsporum gypseum	28	100	100
Trichophyton rubrum	40	100	100
Trichophyton violaceum	50	90	100

oils showed narrow fungitoxic spectrum at lower concentration but broad range of fungitoxicity at higher concentrations. The essential oils were found to exhibit long shelf life because of persistance of their fungitoxicity for 360 days (Table 5). Moreover, when exposed for three hours at

Table 5. Fungitoxicity of *Cinnamomum tamala* and *Citrus maxima* oils stored for different periods.

Stored period (days)	Percent inhibition of mycelial growth			
	Cinnamomum tamala		*Citrus maxima*	
	M. A. (500 ppm)	T. M. (500 ppm)	M. A. (500 ppm)	T. M. (500 ppm)
30	100	100	100	100
60	100	100	100	100
90	100	100	100	100
120	100	100	100	100
150	100	100	100	100
180	100	100	100	100
210	100	100	100	100
240	100	100	100	100
270	100	100	100	100
300	100	100	100	100
330	100	100	100	100
360	100	100	100	100

T. M. = Trichophyton mentagrophytes
M. A. = Microsporum audouinii

different temperatures viz. 25, 30,70, and 100°c, both the oils exhibited thermostable nature of toxicity (Table6). Besides, the oils were found to

Table 6. Fungitoxicity of *Cinnamomum tamala* and *Citrus maxima* oils exposed to different temperatures.

Temperature (°C)	Percent inhibition of mycelial growth			
	Cinnamomum tamala		*Citrus maxima*	
	M. A. (500 ppm)	T. M. (500 ppm)	M. A. (500 ppm)	T. M. (500 ppm)
25	100	100	100	100
50	100	100	100	100
70	100	100	100	100
100	100	100	100	100

M. A. = Microsporum audouinii
T. M. = Trichophyton mentagrophytes

be more efficacious than synthetic antifungal agents viz. clotrimazole, griseofulvin and nystatin and as potent as ketaconazole, benzoic acid and salicylic acid (Table 7).

Table 7. Efficacy of some prevalent antifungal agents against the test dermatophytes.

Concentration (in ppm)	Name of Drug											
	Clotrimazole		Benzoic Acid		Griseofulvin		Ketaconazole		Nystatin		Salicylic Acid	
	M. A.	T. M.	M. A.	T. M.	M. A.	T. M.	M. A.	T. M.	M. A.	T. M.	M. A.	T. M.
500	30	48	100	100	22	20	100	100	20	20	100	100
1000	85	80	100	100	32	28	100	100	25	40	100	100
2000	100	100	100	100	45	50	100	100	100	100	100	100
4000	100	100	100	100	100	100	100	100	100	100	100	100
5000	100	100	100	100	100	100	100	100	100	100	100	100

M. A. = Microsporum audouinii
T. M. = Trichophyton mentagrophytes

DISCUSSION

The study indicates the prospectives of the oils of *Cinnamomum tamala* and *Citrus maxima* against dermatomycosis because of their broad fungitoxic spectrum against ringworm fungi, long shelf life and thermostable nature of biocativity.

Inspite of tremendous development in the field of synthetic drugs during recent era, higher plants still hold their own place as of a source several effective drugs in place of synthetics which have severe side effects. Since most of the essential oils are the isolates of plants which are in use in our traditional medicine, there would be least possiblity of any side effect after their application. Many antifungal compound occur in plants and it is of interest to note that leaves of certain plant species were used for treating fungal infections of man long before the presence of any fungicidal compound was suspected. *Artemisia capillaris* for example, was used for many years by paddy field workers to control fungal infections of feet, has been shown to contain the potent fungicide-capillin (Marini-Bettolo, 1977). Therefore, a systematic approach should be made to find out the efficacy of plants against human pathogenic fungi so as to exploit them as herbal antimicrobial agents. Moreover, the volatile oils (the essential oils) have some prospectives for their application as aroma therapy, for cure of deep mycosis and aspergillosis. However, detailed clinical trials are necessary so as to determine their applicability as herbal antimycotic agents.

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